ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ, ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΕΡΓΑΣΤΗΡΙΟ ΚΛΙΝΙΚΗΣ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ, Π.Γ.Ν. «ΑΤΤΙΚΟΝ»
ΔΙΕΥΘΥΝΤΡΙΑ: ΑΝΑΠΛ. ΚΑΘΗΓΗΤΡΙΑ Λ. ΖΕΡΒΑ

Ανάπτυξη in-vitro Φαρμακοκινητικού/Φαρμακοδυναμικού μοντέλου για την Μελέτη της Δράσης Αντιμυκητιακών Φαρμάκων και των Συνδυασμών τους έναντι ειδών Ασπεργίλλου

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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΑΘΗΝΑ 2013

NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS
SCHOOL OF HEALTH SCIENCES, DEPARTMENT OF MEDICINE

CLINICAL MICROBIOLOGY LABORAOTORY, UNIVERSITY HOSPITAL ATTIKON
THE HEAD: ASS. PROFESSOR L. ZERVA

Development of a new in vitro pharmacokinetic/ pharmacodynamic (PK/PD) model simulating human pharmacokinetics for studying the effect of antifungal drugs and their combination against *Aspergillus* species

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ATHENS 2013

Dedication

To My Family

ΕΘΝΙΚΟΝ & ΚΑΠΟΔΙΣΤΡΙΑΚΟΝ

ΠΑΝΕΠΙΣΤΗΜΙΟΝ ΑΘΗΝΩΝ

ΙΑΤΡΙΚΗ ΣΧΟΛΗ

ΙΠΠΟΚΡΑΤΙΚΟΣ ΟΡΚΟΣ
ΚΕΙΜΕΝΟ

ΟΜΝΥΜΙ ΑΠΟΛΛΩΝΑ ΙΗΤΡΟΝ ΚΑΙ ΑΣΚΛΗΠΙΟΝ ΚΑΙ ΥΓΕΙΑΝ ΚΑΙ ΠΑΝΑΚΕΙΑΝ ΚΑΙ ΘΕΟΥΣ ΠΑΝΤΑΣ ΤΕ ΚΑΙ ΠΑΣΑΣ ΙΣΤΟΡΑΣ ΠΟΙΕΥΜΕΝΟΣ, ΕΠΙΤΕΛΕΑ ΠΟΙΗΣΕΙΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ ΟΡΚΟΝ ΤΟΝΔΕ ΚΑΙ ΞΥΓΓΡΑΦΗΝ ΤΗΝΔΕ. ΗΓΗΣΕΣΘΑΙ ΜΕΝ ΤΟΝ ΔΙΔΑΞΑΝΤΑ ΜΕ ΤΗΝ ΤΕΧΝΗΝ ΤΑΥΤΗΝ ΙΣΑ ΓΕΝΕΤΗΣΙΝ ΕΜΟΙΣΙ, ΚΑΙ ΒΙΟΥ ΚΟΙΝΩΣΕΣΘΑΙ ΚΑΙ ΧΡΕΩΝ ΑΠΟΧΡΙΖΟΝΤΙ ΜΕΤΑΔΟΣΙΝ ΠΟΙΗΣΕΣΘΑΙ ΚΑΙ ΓΕΝΟΣ ΤΟ ΕΞ ΑΥΤΟΥ ΑΔΕΛΦΕΟΙΣ ΙΣΟΝ ΕΠΙΚΡΙΝΕΕΙΝ ΑΡΡΕΣΙ ΚΑΙ ΔΙΔΑΞΕΙΝ ΤΗΝ ΤΕΧΝΗΝ ΤΑΥΤΗΝ, ΗΝ ΧΡΗΙΖΩΣΙ ΜΑΝΘΑΝΕΙΝ, ΑΝΕΥ ΜΙΣΘΟΥ ΚΑΙ ΞΥΓΓΡΑΦΗΣ ΠΑΡΑΓΓΕΛΙΗΣ ΤΕ ΚΑΙ ΑΚΡΟΗΣΙΟΣ ΚΑΙ ΤΗΣ ΛΟΙΠΗΣ ΑΠΑΣΗΣ ΜΑΘΗΣΙΟΣ ΜΕΤΑΔΟΣΙΝ ΠΟΙΗΣΕΣΘΑΙ ΥΙΟΙΣΙ ΤΕ ΕΜΟΙΣΙ ΚΑΙ ΤΟΙΣΙ ΤΟΥ ΕΜΕ ΔΙΔΑΞΑΝΤΟΣ ΚΑΙ ΜΑΘΗΤΑΙΣΙ ΞΥΓΓΕΓΡΑΜΜΕΝΟΙΣΙ ΤΕ ΚΑΙ ΟΡΚΙΣΜΕΝΟΙΣ ΝΟΜΩ ΙΗΤΡΙΚΩ ΑΛΛΩ ΔΕ ΟΥΔΕΝΙ. ΔΙΑΙΤΗΜΑΣΙ ΤΕ ΧΡΗΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ ΚΑΤΑ ΔΥΝΑΜΙΝ ΚΑΙ ΚΡΙΣΙΝ ΕΜΗΝ, ΕΠΙ ΔΗΛΗΣΕΙ ΔΕ ΚΑΙ ΑΔΙΚΙΗ/ΕΙΡΞΕΙΝ ΤΟΥ ΙΚΩΣΤΑΔΕ ΟΥΔΕ ΦΑΡΜΑΚΟΝ ΟΥΔΕΝΙ ΑΙΤΗΘΕΙΣ ΘΑΝΑΣΙΜΟΝ, ΟΥΔΕ ΥΦΗΓΗΣΟΜΑΙ ΞΥΜΒΟΥΛΙΗΝ ΤΟΙΗΝΔΕ· ΟΜΟΙΩΣ ΔΕ ΟΥΔΕ ΓΥΝΑΙΚΙ ΠΕΣΣΟΝ ΦΘΟΡΙΟΝ ΔΩΣΩ. ΑΓΝΩΣ ΔΕ ΚΑΙ ΟΣΙΩΣ ΔΙΑΤΗΡΗΣΩ ΒΙΟΝ ΤΟΝ ΕΜΟΝ ΚΑΙ ΤΕΧΝΗΝ ΤΗΝ ΕΜΗΝ. ΟΥ ΤΕΜΕΩ ΔΕ ΟΥΔΕ ΜΗ ΛΙΘΩΝΤΑΣ, ΕΚΧΩΡΗΣΩ ΔΕ ΕΡΓΑΤΗΣΙΝ ΑΝΔΡΑΣΙΝ ΠΡΗΞΙΟΣΤΗΣΙΝ ΤΕΣΣΙΝ ΙΣΙΝ. ΟΙΚΙΑΣ ΔΕ ΟΚΟΣΑΣ ΑΝ ΕΣΙΩ, ΕΣΕΛΕΥΣΟΜΑΙ ΕΠ' ΩΦΕΛΕΙΗ ΚΑΜΝΟΝΤΩΝ, ΕΚΤΟΣ ΕΩΝ ΠΑΣΗΣ ΑΔΙΚΙΗΣ ΕΚΟΥΣΙΗΣ ΚΑΙ ΦΘΟΡΙΗΣ ΤΗΣ ΤΕ ΑΛΛΗΣ ΚΑΙ ΑΦΡΟΔΙΣΙΩΝ ΕΡΓΩΝ ΕΠΙ ΤΕ ΓΥΝΑΙΚΕΙΩΝ ΣΩΜΑΤΩΝ ΚΑΙ ΑΝΔΡΕΙΩΝ, ΕΛΕΥΘΕΡΩΝ ΤΕ ΚΑΙ ΔΟΥΛΩΝ. Α Δ' ΑΝ ΕΝ ΘΕΡΑΠΕΙΗ Η ΙΔΩ Η ΑΚΟΥΣΩ, Η ΚΑΙ ΑΝΕΥ ΘΕΡΑΠΕΙΗΣ ΚΑΤΑ ΒΙΟΝ ΑΝΘΡΩΠΩΝ, Α ΜΗ ΧΡΗ ΠΟΤΕ ΕΚΛΑΛΕΕΣΘΑΙ ΕΞΩ, ΣΙΓΗΣΟΜΑΙ ΑΡΡΗΤΑ ΗΓΕΥΜΕΝΟΣ ΕΙΝΑΙ ΤΑ ΤΟΙΑΥΤΑ. ΟΡΚΟΝ ΜΕΝ ΟΥΝ ΜΟΙ ΤΟΝΔΕ ΕΠΙΤΕΛΕΑ ΠΟΙΕΟΝΤΙ ΚΑΙ ΜΗ ΞΥΓΧΕΟΝΤΙ ΕΙΗ ΕΠΑΥΡΑΣΘΑΙ ΚΑΙ ΒΙΟΥ ΚΑΙ ΤΕΧΝΗΣ, ΔΟΞΑΖΟΜΕΝΩ ΠΑΡΑ ΠΑΣΙΝ ΑΝΘΡΩΠΟΙΣ ΕΣ ΤΟΝ ΑΙΕΙ ΧΡΟΝΟΝ· ΠΑΡΑΒΑΙΝΟΝΤΙ ΔΕ ΚΑΙ ΕΠΙΟΡΚΕΟΝΤΙ ΤΑΝΑΝΤΙΑ ΤΟΥΤΕΩΝ.

ΙΠΠΟΚΡΑΤΗΣ

ΙΠΠΟΚΡΑΤΙΚΟΣ ΟΡΚΟΣ
ΜΕΤΑΦΡΑΣΗ

ΟΡΚΙΖΟΜΑΙ ΣΤΟΝ ΑΠΟΛΛΩΝΑ ΤΟΝ ΙΑΤΡΟ ΚΑΙ ΣΤΟΝ ΑΣΚΛΗΠΙΟ ΚΑΙ ΣΤΗΝ ΥΓΕΙΑ ΚΑΙ ΣΤΗΝ ΠΑΝΑΚΕΙΑ ΚΑΙ ΣΕ ΟΛΟΥΣ ΤΟΥΣ ΘΕΟΥΣ ΚΑΙ ΤΙΣ ΘΕΕΣ, ΠΟΥ ΒΑΖΩ ΜΑΡΤΥΡΕΣ ΚΑΙ ΘΑ ΕΚΠΛΗΡΩΣΩ ΤΟΝ ΟΡΚΟ ΜΟΥ ΑΥΤΟ ΚΑΙ ΤΟ ΣΥΜΒΟΛΑΙΟ ΑΥΤΟ ΣΥΜΦΩΝΑ ΜΕ ΤΗ ΔΥΝΑΜΗ ΜΟΥ ΚΑΙ ΤΗΝ ΚΡΙΣΗ ΜΟΥ. ΟΤΙ ΘΑ ΘΕΩΡΩ ΕΚΕΙΝΟΝ ΠΟΥ ΜΟΥ ΔΙΔΑΞΕ ΤΗΝ ΤΕΧΝΗ ΑΥΤΗ ΙΣΟΝ ΜΕ ΤΟΥΣ ΓΟΝΕΙΣ ΜΟΥ, ΚΑΙ ΘΑ ΤΟΝ ΚΑΝΩ ΚΟΙΝΩΝΟ ΤΟΥ ΒΙΟΥ ΜΟΥ, ΚΑΙ ΘΑ ΤΟΥ ΠΡΟΣΦΕΡΩ ΑΠΟ ΤΑ ΔΙΚΑ ΜΟΥ ΟΤΙ ΧΡΕΙΑΖΕΤΑΙ. ΤΟΥΣ ΣΠΟΓΟΝΟΥΣ ΤΟΥ ΘΑ ΘΕΩΡΩ ΩΣ ΑΔΕΛΦΟΥΣ ΜΟΥ ΚΑΙ ΘΑ ΤΟΥΣ ΔΙΔΑΞΩ ΤΗΝ ΤΕΧΝΗ ΑΥΤΗ, ΑΝ ΕΠΙΘΥΜΟΥΝ ΝΑ ΜΑΘΟΥΝ, ΧΩΡΙΣ ΜΙΣΘΟ ΚΑΙ ΧΩΡΙΣ ΣΥΜΦΩΝΙΑ. ΟΤΙ ΘΑ ΜΕΤΑΔΩΣΩ ΤΟΥΣ ΕΠΑΓΓΕΛΜΑΤΙΚΟΥΣ ΚΑΝΟΝΕΣ, ΤΑ ΘΕΩΡΗΤΙΚΑ ΜΑΘΗΜΑΤΑ ΚΑΙ ΤΙΣ ΥΠΟΛΟΙΠΕΣ ΔΙΑΦΟΡΕΣ ΑΣΚΗΣΕΙΣ ΣΤΟΥΣ ΠΟΥΣ ΜΟΥ, ΣΤΟΥΣ ΠΟΥΣ ΤΟΥ ΔΙΔΑΣΚΑΛΟΥ ΜΟΥ ΚΑΙ ΣΕ ΜΑΘΗΤΕΣ ΠΟΥ ΘΑ ΕΧΟΥΝ ΣΥΝΔΕΘΗ ΜΑΖΙ ΜΟΥ ΜΕ ΟΡΚΟ ΚΑΙ ΣΥΜΒΟΛΑΙΟ, ΚΑΤΑ ΤΗ ΣΥΝΗΘΕΙΑ ΤΩΝ ΙΑΤΡΩΝ, ΚΑΙ ΣΕ ΚΑΝΕΝΑ ΑΛΛΟ.

ΘΑ ΧΡΗΣΙΜΟΠΟΙΗΣΩ ΤΗ ΘΕΡΑΠΕΥΤΙΚΗ ΔΙΑΤΑΣΗ ΜΟΝΟ ΓΙΑ ΩΦΕΛΕΙΑ ΤΩΝ ΑΡΡΩΣΤΩΝ, ΟΣΟ ΒΡΙΣΚΟΜΑΙ ΑΠΟ ΤΗ ΔΥΝΑΜΗ ΚΑΙ ΤΗΝ ΚΡΙΣΗ ΜΟΥ, ΚΑΙ (ΥΠΟΣΧΟΜΑΙ ΟΤΙ) ΘΑ ΤΟΥΣ ΠΡΟΦΥΛΑΞΩ ΑΠΟ ΚΑΘΕ ΒΛΑΒΗ ΚΑΙ ΑΔΙΚΙΑ.

ΔΕΝ ΘΑ ΧΟΡΗΓΗΣΩ ΘΑΝΑΤΗΦΟΡΟ ΦΑΡΜΑΚΟ ΣΕ ΚΑΝΕΝΑ, ΟΣΟ ΚΑΙ ΑΝ ΠΑΡΑΚΛΗΘΩ. ΟΥΤΕ ΘΑ ΥΠΟΔΕΙΞΩ ΤΕΤΟΙΑ ΣΥΜΒΟΥΛΗ. ΕΠΙΣΗΣ ΔΕΝ ΘΑ ΔΩΣΩ ΣΕ ΓΥΝΑΙΚΑ ΦΑΡΜΑΚΟ ΕΚΤΡΩΤΙΚΟ. ΑΓΝΗ ΚΑΙ ΚΑΘΑΡΗ ΘΑ ΔΙΑΤΗΡΗΣΩ ΤΗ ΖΩΗ ΜΟΥ ΚΑΙ ΤΗΝ ΤΕΧΝΗ ΜΟΥ. ΔΕΝ ΘΑ ΧΕΙΡΟΥΡΓΗΣΩ ΟΠΟΣΔΗΠΟΤΕ ΑΤΟΜΟ ΠΟΥ ΠΑΣΧΟΥΝ ΑΠΟ ΠΕΤΡΑ, ΑΛΛΑ ΘΑ ΑΦΗΣΩ ΤΗΝ ΠΡΑΞΗ ΑΥΤΗ ΣΤΟΥΣ ΕΞΑΣΚΗΜΕΝΟΥΣ.

ΣΕ ΟΣΑ ΣΠΙΤΙΑ ΠΡΟΣΚΑΛΟΥΜΑΙ, ΘΑ ΜΠΑΙΝΩ ΓΙΑ ΤΟ ΚΑΛΟ ΤΩΝ ΑΡΡΩΣΤΩΝ, ΚΡΑΤΩΝΤΑΣ ΜΑΚΡΙΑ ΤΟΝ ΕΑΥΤΟ ΜΟΥ ΜΑΚΡΙΑ ΑΠΟ ΚΑΘΕ ΘΕΛΗΜΑΤΙΚΗ ΑΔΙΚΙΑ ΚΑΙ ΑΛΛΗ ΔΙΑΦΘΟΡΑ ΚΑΙ ΠΡΟ ΠΑΝΤΩΝ ΜΑΚΡΙΑ ΑΠΟ ΚΑΘΕ ΑΦΡΟΔΙΣΙΑΚΗ ΠΡΑΞΗ ΣΕ ΣΩΜΑΤΑ ΓΥΝΑΙΚΩΝ ΚΑΙ ΑΝΔΡΩΝ, ΕΛΕΥΘΕΡΩΝ Η ΔΟΥΛΩΝ.

ΟΣΑ ΔΕ ΚΑΤΑ ΤΗΝ ΔΙΑΡΚΕΙΑ ΤΗΣ ΘΕΡΑΠΕΙΑΣ ΘΑ ΔΩ Η ΘΑ ΑΚΟΥΣΩ, Η ΚΑΙ ΠΕΡΑ ΑΠΟ ΤΙΣ ΑΣΧΟΛΙΕΣ ΜΟΥ ΣΤΗΝ ΚΑΘΗΜΕΡΙΝΗ ΖΩΗ, ΟΣΑ ΔΕΝ ΠΡΕΠΕΙ ΠΟΤΕ ΝΑ ΚΟΙΝΟΛΟΓΟΥΝΤΑΙ ΣΤΟΥΣ ΕΞΩ, ΘΑ ΤΑ ΑΠΟΣΙΩΠΩ. ΥΠΟΛΟΓΙΖΟΝΤΑΣ ΟΤΙ ΑΥΤΑ ΕΙΝΑΙ ΙΕΡΑ ΜΥΣΤΙΚΑ. ΟΣΟ ΛΟΙΠΟΝ ΘΑ ΤΗΡΩ ΤΟΝ ΟΡΚΟ ΜΟΥ ΑΥΤΟ, ΚΑΙ ΔΕΝ ΘΑ ΤΟΝ ΠΑΡΑΒΙΑΖΩ, ΕΙΘΕ ΝΑ ΠΕΤΥΧΑΙΝΩ ΣΤΗ ΖΩΗ ΚΑΙ ΣΤΗΝ ΤΕΧΝΗ ΜΟΥ, ΕΧΟΝΤΑΣ ΚΑΛΟ ΟΝΟΜΑ ΠΑΝΤΟΤΕ ΑΝΑΜΕΣΑ ΣΤΟΥΣ ΑΝΘΡΩΠΟΥΣ· ΕΑΝ ΟΜΩΣ ΤΟΝ ΠΑΡΑΒΩ ΚΑΙ ΓΙΝΩ ΕΠΙΟΡΚΟΣ, ΝΑ ΠΑΘΩ ΤΑ ΑΝΤΙΘΕΤΑ.

Submission Dates:

- Submission date for Ph.D. study at Medical School of Athens: 1/6/2009 – Protocol No. 3778

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1. Meletiadis J, **Al-Saigh R**, Velegraki A, Walsh TJ, Roilides E, Zerva L. Pharmacodynamic effects of simulated standard doses of antifungal drugs against Aspergillus species in a new in vitro pharmacokinetic/pharmacodynamic model. *Antimicrob Agents Chemother*. 2012; 56(1):403-10.
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II. J. Meletiadis, **R. Al-Saigh**, L. Zerva. Amphotericin B, voriconazole and caspofungin activity against Aspergillus fumigatus in an in vitro pharmacokinetic/pharmacodynamic (PK/PD) diffusion system simulating human pharmacokinetics. 4th Trends in Medical Mycology Abstr. no.: 283. October 2009, Athens, Greece.

III. Ιωσήφ Μελετιάδης, **Rafal Al-Saigh**, Λουκία Ζέρβα. Ανάπτυξη ενός νέου φαρμακοκινητικού/φαρμακοδυναμικού συστήματος που προσομοιώνει την φαρμακοκινητική της αμφοτερικίνης στον άνθρωπο για την μελέτη της δραστικότητας της έναντι ειδών ασπεργίλλου. Υποβλήθηκε για το Συνέδριο Ελληνικής Εταιρίας Ιατρικής Μυκητολογικός,2010, Αθήνα.

IV. J. Meletiadis, **R. Al-Saigh**, L. Zerva. Comparative pharmacodynamic characteristics of amphotericin B against Aspergillus fumigatus, A. flavus and A. terreus using an in vitro pharmacokinetic/pharmacodynamic system simulating amphotericin human pharmacokinetics. 20th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria, 10-13 April 2010.

V. **R. Al-Saigh**, L. Zerva J. Meletiadis. The study of voriconazole activity against Aspergillus species in an in vitro pharmacokinetic/pharmacodynamic (PK/PD) diffusion system simulating human pharmacokinetics. 6^{o} Πανελλήνιο Συνέδριο Ιατρική Βιοπαθολογίας, Αθήνα, 2010.

VI. Ιωσήφ Μελετιάδης, **Rafal Al-Saigh**, Λουκία Ζέρβα. Ανάπτυξη ενός in vitro φαρμακοκινητικού/φαρμακοδυναμικού συστήματος για την μελέτη της δραστικότητας αντιμυκητιασικών φαρμάκων έναντι ασπεργίλλων. «***Βραβείο Σ. Παπασταματη», 36ο Πανελλήνιο Ιατρικό Συνέδριο,*** 4-8 Μαΐου, Αθηνα, 2010.

VII. J. Meletiadis, **R. Al-Saigh**, A. Elefanti, L. Zerva. InVitro Pharmacokinetic/ pharmacodynamic (PKPD) Modeling Of Voriconazole (VOR) Activity Against Aspergillus (ASP) Species In a New In Vitro PK System. 50th ICAAC-2010, Boston-USA.

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IX. Μ. Σιώπη, Α. Ελεφάντη, **Rafal Al-Saigh**, Ν. Σιαφάκα, Λουκία Ζέρβα Ιωσήφ Μελετιάδης. Προσδιορισμός ορίων ευαισθησίας βορικοναζόλης για *Aspergillus fumigatus* με ένα in vitro δυναμικό μοντέλο φαρμακοκινητικής προσομοίωσης. «***Έπαθλο Σ. Παπασταματη», 38ο Πανελλήνιο Ιατρικό Συνέδριο,*** 19 Μαΐου, Αθήνα, 2012.

Lectures within scientific conferences:

- **R. Al-Saigh**, L. Zerva J. Meletiadis. The study of voriconazole activity against Aspergillus species in an in vitro pharmacokinetic/pharmacodynamic (PK/PD) diffusion system simulating human pharmacokinetics. 6ο Πανελλήνιο Συνέδριο Ιατρική Βιοπαθολογίας, Αθήνα, 2010.

Languages:

- English: (good grade in reading, writing and conversation).
- A certificate in Modern Greek Language June-2009/ Athens/Greece.
- Arabic (native language)

CONTENTS

List of abbreviation

ABCD	Amphotericin B Colloidal Dispersion
ABLC	Amphotericin B lipid Complex
ABPA	Allergic BrochoPulmonary Aspergillosis
AFST	Antifungal Susceptibility Testing
AMB	Amphotericin B
AUC	Area Under the Curve
CE	Conidial Equivalent
CFU	Colonies F Unites
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Maximum Concentration
CT	Threshold Cycle
CYP2C19	Cytochrome P-450 Isoenzyme
D-AMB	Sodium Deoxycholate Amphotericin B
EC	External ompartment
E_{max}	The Maximum Galactomannan Ratio
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FIC	Fractional Inhibitory Concentration
GI	Galactomannan index
HEPA	High-Efficiency Particulate Air
HSCT	haemopoietic stem cell transplantation
IA	Invasive aspergillosis
IC	Internal compartment
IPA	Invasive Pulmonary Apergillosis
L-AMB	Liposomal Amphotericin B
MEC	Minimal Effective Concentration
MIC	Minimal Inhibitory Concentration
MSG/EORTC	Mycoses Study Group/European Organization for Research and Treatment of Cancer
PAFE	Post Antifungal Effect
PD	Pharmacodynamic
PK	Pharmacokinetic
$t_{1/2}$	Half life
T_{50}	Time corresponding to 50% of E_{max}
T_o	Independent Variable
VOR	Voriconazole
Γ	The Slope of the Curve
%T	Target Percent Transmission
5-FC	5- Fluorocytosine

Preface

Aspergillosis presents a large spectrum of clinical manifestation caused by members of the genus *Aspergillus* ranging from allergic, chronic, and saprophytic conditions to invasive diseases (Patterson et al, 2000). Invasive aspergillosis (IA) currently constitutes the most common cause of infectious pneumonic mortality in patients undergoing hematopoietic stem cell transplantation (Marr et al, 2002), and is an important cause of opportunistic respiratory and disseminated infection in other immunocompromised patients as in solid organ transplantation recipients, especially in lung transplantation patients (Walsh et al, 2008). *Aspergillus* is a large genus, containing over 200 species, to which humans are continuously exposed. However, only a small number of these species have been associated with invasive infections, and over 90% of all infections are caused by no more than three species: *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus terreus* (Steinbach et al, 2012). Antifungal drugs used to treat these infections belong to three classes; the polyenes like amphotericin B which acts on cell membrane, the azoles like voriconazole, itraconazole and posaconazole which inhibit ergosterol biosynthesis pathways and echinocandins like caspofungin, anidulafungin and micafungin which inhibit 1,3-β-D glucan synthase and cell wall biosynthesis.

In vitro susceptibility testing of *Aspergillus* species against amphotericin B, voriconazole, and caspofungin has been standardized providing useful information about the in vitro activity of antifungal drugs (CLSI, 2008). The in vitro activities of antifungal agents against *Aspergillus* spp. are usually determined by standardized broth microdilution assays where the minimum inhibitory concentration (MIC) (for azoles and amphotericin B) or minimum effective concentration (MEC; for echinocandins) is defined as the lowest drug concentration that completely inhibits fungal growth or causes typical morphological changes of the fungus, respectively (CLSI, 2008). These endpoints are obtained using assays where the drug concentration remains constant over time (Cantón et al, 2009). However, fungi are exposed in vivo to fluctuating drug concentrations over time because of absorption, elimination, excretion, metabolism, and distribution processes (Groll et al, 1998). The impact of fluctuating drug concentrations on *Aspergillus* growth is unknown and might be different from the effect of constant drug concentrations over time (Li and Zhu, 1997). Also, several factors may influence clinical outcome related to host

(underlying condition, immunosuppression), drug (timing of administration, suboptimal exposure) and pathogen (resistance, virulence). As most *Aspergillus* species have low resistance rates to antifungal agents, we cannot explain the high mortality associated with these infections (Mayr et al, 2011; Snelders et al, 2011). Furthermore, the most frequently isolated species, *A. fumigatus, A. flavus* and *A. terreus* demonstrate similar in vitro susceptibility although in vivo experimental and clinical data show that the efficacy of antifungal agents differ for these species (Steinbach et al, 2004; Takemoto et al, 2009; Walsh et al, 2008; Warn et al, 2006). Furthermore, amphotericin B (AMB) is a highly lipophilic and amphoteric molecule that offers a wide spectrum of antifungal activity and fungicidal action that it exerts a range of different pharmacodynamic effects from fungicidal to fungistatic actions, post antifungal effect, concentration-dependent killing, and sub-MIC effect beyond its inhibitory effect (Dodds et al, 2000).

Moreover, a variety of important pharmacokinetic/pharmacodynamic (PK/PD) indices (C_{max}/MIC, T>MIC, AUC/MIC) cannot be studied in vitro against *Aspergillus* spp. since MIC testing by standard methodology does not produce clinically relevant drug exposures (Andes, 2006). In vitro models that simulate the human plasma pharmacokinetics of antifungal drugs have been developed for *Candida* species, enabling the study of the effects of clinically relevant drug concentrations on different *Candida* species (Lewis et al, 2002, Lewis et al, 2006). Furthermore, they have proved to be very useful in investigating the efficacy of antifungal drug combinations against *Candida* spp. (Lewis et al, 1998, Lignell et al, 2007). The development of similar models for *Aspergillus* spp. has been hampered so far because their filamentous growth, which makes it difficult to determine fungal growth.

1.1. Fungi and fungal infections

Fungi are a diverse group of organisms. Most fungi are microscopic, but some are macroscopic, particularly the fruiting bodies of mushrooms. Fungi are common in the environment and have several key roles in the functioning of ecosystems (Newbound et al, 2010). The estimated number of fungal species is at least 1.5 million, although only 72,000 have been formally described (Hawksworth, 2001). Many fungi play key roles in recycling of nutrients through decomposition of dead plant biomass and bioremediation (Barr and Aust, 1994). Some species form symbiotic relationships with plants (Rodriguez and Redman, 2008) which are essential for their survival in terrestrial environments. Some fungi are food sources for humans or animals and others are used in industrial fermentation processes to produce useful biochemicals (Bennett, 1998). However, some fungi are harmful for plants, animals and humans (Monk and Goffeau, 2008; Cannon et al., 2009). About 300 to 400 fungi are reported to be pathogenic to humans and to cause a wide variety of diseases (Person et al., 2010; Moran et al., 2011). Fungal infections can be acquired by inhalation, traumatic inoculation or endogenous sources. Fungi can cause superficial infections of skin and mucous membranes of healthy individuals, as well as subcutaneous infections that can be painful and disfiguring. Fungi can also penetrate tissues or into the blood system of immunocompromised patients to cause invasive and disseminated infections which are associated with high morbidity and mortality (Menzin et al., 2009).

Fungi are eukaryotic, heterotrophic microorganisms. Fungi and animals are in neighboring kingdoms in the domain Eukarya. An important difference between these two groups is that fungal cells are supported externally by a strong and flexible cell wall, whereas animal cells are not. Regarding their growth form, fungi can be multicellular (filamentous), unicellular (yeasts) or dimorphic (able to switch between unicellular and multicellular forms) (Osiewacz, 2002). Filamentous fungi (moulds) are composed of long tubular cells called hyphae that exhibit tip localized growth (Kaminskyj and Heath, 1996). Hyphae may be divided by cross walls (septa), although certain fungal groups are aseptate. Major groups of septate fungi are the Ascomycetes (e.g. *Aspergillus* and *Candida*) and the Basidiomycetes (e.g. *Cryptococcus*). The major aseptate group of filamentous fungi is the Zygomycetes (e.g. *Rhizopus* spp., *Mucor* spp.). Hyphae of septate

filamentous fungi can be uninucleate or multinucleate. In *Aspergillus*, the nuclei are typically haploid, except before sexual reproduction or during parasexual cycle.

Most filamentous fungi produce specialized cells for dispersal, called conidia. Fungal conidia contain one or more nuclei and mitochondria, have limited nutrient reserves, and in many species they can survive long periods of dormancy. Many conidia are airborne i.e. dispersed by air. Conidia are asexual propagules (reproductive units) whereas spores may be either sexual or asexual. Asexual spores are produced in sac-like cells called sporangia and are called sporangiospores. Sexual spores include ascospores, basidiospores, oospores, and zygospores, which are used to determine phylogenetic relationships. Sexual spores are produced by meiosis whereas asexual spores are produced by mitosis (Taylor et al., 1999). For many filamentous fungi including *Aspergillus* the predominant life cycle stage is asexual (Wearing, 2010). In addition, most filamentous fungi can produce colonies from hyphal fragments (vegetative reproduction). Filamentous fungi acquire nutrients during vegetative growth, forming mycelial colonies that produce spores for dispersal, as well as being able to regrow from fragments if a colony is physically damaged.

The clinical nomenclatures used for the infections caused by fungi (mycoses) are based on the site of the infection (superficial, cutaneous, deep infections), route of acquisition of the pathogen (exogenous, endogenous) and type of virulence exhibited by the fungus (primary or opportunistic pathogens). Opportunistic fungal infections cause a variety of symptoms depending on the species, type of infection and the affected area of the body. Persons at risk for fungal infections include: 1) individual with immunodeficinces, 2) the very young and the very old, 3) diabetic patients, 4) patients taking steroid medication or antibacterial antibiotics for extended periods of time, 5) patients with underlying diseases like HIV and CMV infection and 6) patients on chemotherapy and transplantation regimes (Ringden et al., 1991; Perlroth et al., 2007).

Fungal infections Fungal infections may be superficial (limited to the stratum corneum), cutaneous (involve the integument and its appendages), subcutaneous (include a range of different infections characterized by infection of the subcutaneous tissues usually at the point of traumatic inoculation), or systemic, deep (involve the lungs, abdominal viscera, bones and/or central nervous system) infections depending on the type

and degree of tissue involvement and the host's response to the pathogen. The most common portals of entry are the respiratory and gastrointestinal tract.

1.2. Aspergillus

The opportunistic mould *Aspergillus* is the etiologic agent responsible for a variety of infections and conditions referred to as aspergillosis.

Aspergillus is a genus of filamentous fungi that has more than 200 species. *Aspergillus* has diverse ecological roles. Its habitat includes soil, dust, and living or dead plant materials (Latge and Steinbach, 2009). The genus *Aspergillus* includes human and plant pathogens as well as beneficial species used to produce foodstuffs and industrial enzymes. For example, *A. fumigatus* is a life-threaening pathogen of immunocompromised patients; *A. flavus* is an agriculturally important toxin producer; *A. niger* and *A. oryzae* are used in industrial processes (Kapoor et al., 1999).

The name of the genus was given by Pietro Antonio Micheli in Florence in 1729 (Micheli, 1729), when looking into the microscope the shape of conidiophores reminded him of the aspergillum (holy water sprinkler) (Raper and Fennel 1965). The potential for allergic reactions to *Aspergillus* in the form of allergic bronchopulmonary aspergillosis was described in 1952 (Hinson et al, 1952). It was not until the mid-1950s with the introduction of immunosuppressive agents such as corticosteroids and cytotoxic chemotherapy that the first occurrences of invasive aspergillosis in immunocompromised hosts were recognized (Rankin, 1953). In recent decades the use of immunosuppressive therapies in increasing numbers of patients has resulted in a dramatic global increase in cases of invasive infections due to Aspergillus (Groll et al, 1996).

Macroscopically the strains are quite diverse; their color varies from white, yellow, blue-green till black, depending on the species and sometimes the age and growth conditions. Microscopically, the Aspergilli show some common features: hyphae are septate and hyaline and the conidiophores terminate in a vesicle at the apex (figure 1.1). This vesicle is a typical organ for Aspergilli. The ascomycete *Aspergillus* genus contains many different fungi, which are mostly saprobic, but human and plant pathogens can be found in this genus, together with strains widely used in industry. So far 114 out of 186 *Aspergillus* species were found to produce only asexual mitotic conidia, like *A. flavus*, *A. fumigatus* and *A. terreus*. The other 72 species produce sexual meiotic ascospores. These

sexual Aspergilli mostly belong to nine genera, including *Emericella*, *Eurotium* and *Neosartorya*. Phylogenetic analysis of 15 species revealed that sexual and asexual Aspergilli are closely related, and meiosis was independently lost and/or gained at least four times in *Aspergillus* among the sampled taxa. In addition, asexual lineages are derived from sexual meiotic ancestors, and data do not support the existence of ancient asexual lineages (Geiser et al, 1996). Modern taxonomy of Aspergilli is an ongoing science which currently groups *Aspergillus* species in 8 subgenera with several sections, members of which are indistinguishable morphologically requiring polyphasic approaches including DNA sequencing for final species identification (Samson et al, 2006).

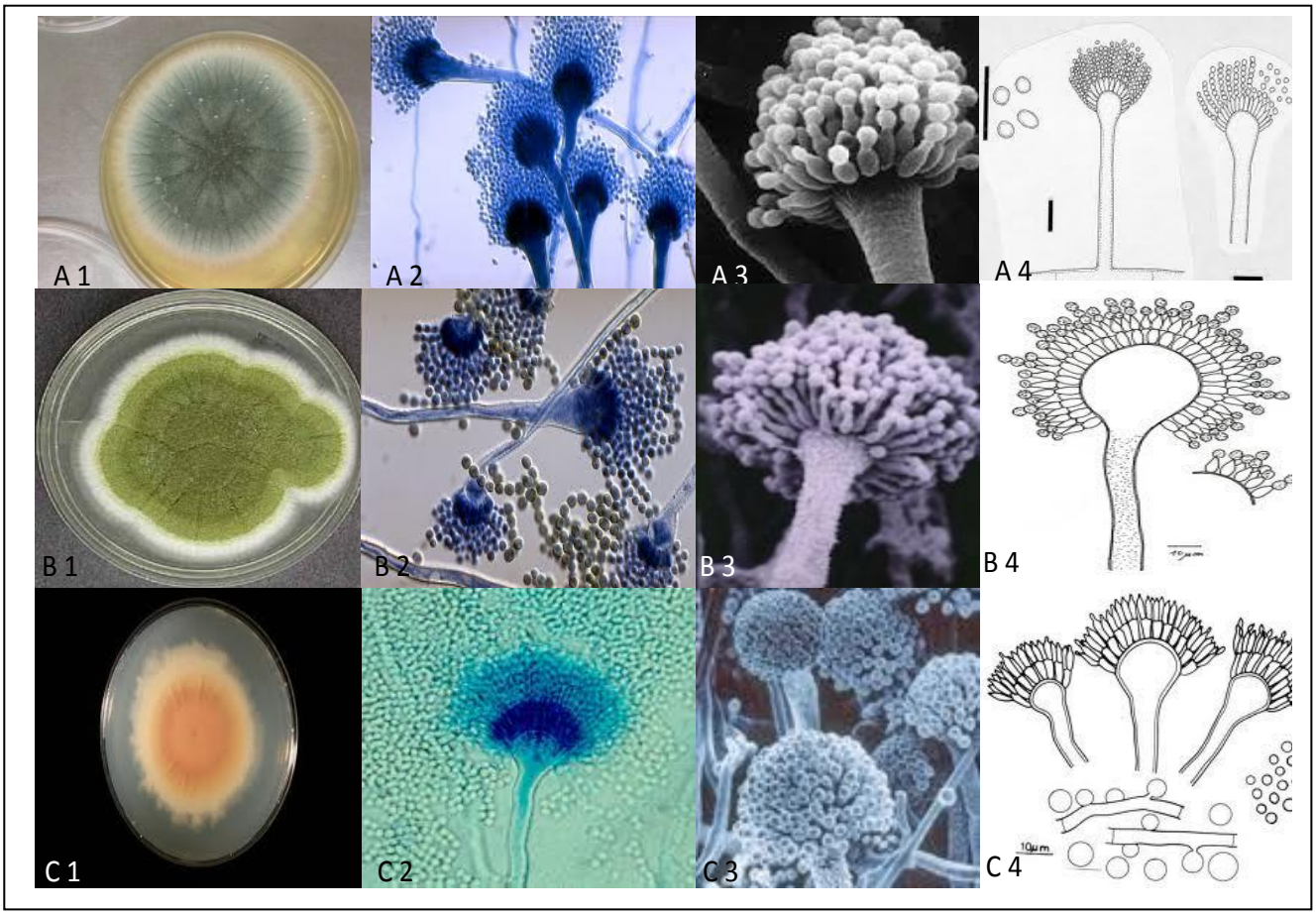

Figure 1.1 Representative macroscopic (1) and microscopic features (2- stained, 3- electron microscope, 4- drawing) of three Aspergillus species (A) - *A. fumigatus*, (B)-*A. flavus*, (C)-*A. terreus*)

Lack of sex may force a fungus to use an alternate pathway of recombination, the so called parasexual cycle (Figure 1.2). In the sexual life cycle reproduction is based on meiotically produced ascospores, whereas in the asexual life cycle mitotically produced conidia are responsible for inheritance. If strains are heterokaryon compatible they may

fuse to form heterokaryons and heteroplasmons in which recombination may occur (parasex). This incompatibility blocks the transfer of genetic elements between different isolates and can be visualised as the inability to form heterokaryons on selective media (van Diepeningen et al, 1997). Barrages as seen in *Podospora anserina* or the formation of aerial hyphae are not visible in these incompatible combinations.

After the initial early descriptions of the organism, *Aspergillus flavus* was formally named in 1809 (Link, 1809). Thom and Church first classified the genus in 1926 with 69 *Aspergillus* species in 11 groups which has gradually increased to over 200 species (although some are not universally accepted) in 18 groups (Thom and Church, 1926; Raper and Fennel, 1965; Pitt, 1994; Denning, 2000). Most species of *Aspergillus* reproduce asexually are named based on asexual reproduction (anamorph) but a teleomorph (or sexual form) has been identified for some species. The generic name *Aspergillus* is generally applied to all species regardless of their teleomorphs, rather than separating the organisms into a limited number of new (and unfamiliar) species based on discovery of a sexual state along with the majority of isolates without an identified teleomorph (Kwon-Chung, 1987). The genus *Aspergillus* is classified in the family Moniliaceae of the form class Hyphomycetes in the phylum Deuteromycota. The teleomorphs of *Aspergillus* species are classified in 10 genera in the phylum Ascomycota. Specific species identification of the organism may be difficult and taxonomy of the species is undergoing revision with the use of molecular tools of identification. *Aspergillus* is distinct but is closely related to the genus *Penicillium* (Klich and Pitt, 1988).

The most common species causing invasive infection include: *Aspergillus fumigatus*, *A. flavus* and *A. terreus*. Recent studies have shown continued emergence of less common species, including *A. terreus* and unusual less pathogenic species as the etiologic agents of invasive infection. With more prolonged and profound immunosuppression, the list of rare species causing invasive infection continues to increase and includes: *A. niger, A. amstelodami*, *A. avenaceus*, *A. caesiellus*, *A. candidus*, *A. carneus*, *A. chevalieri*, *A. clavatus*, *A. flavipes*, *A. glaucus*, *A. granulosus*, *A. nidulans* (*A. nidulellus*), *A. oryzae*, *A. quadrilineatus*, *A. restrictus*, *A. sydowii*, *A. ustus*, *A. versicolor*, *A. wentii*, *Neosartorya pseudofisheri* (previously *Aspergillus fischeri*), and others (Denning, 2000; Klich and Pitt, 1988; Kwon-Chung and Bennett, 1992; Denning, 1998; Stevens et al, 2000; Sutton et al, 1998; Sutton et al, 2000).

Pathogenic *Aspergillus* species grow easily and rapidly at a broad range of culture temperatures and on a wide variety of media, although blood cultures are uncommonly positive. Growth of pathogenic species at 37°C is a feature that differentiates them from non-pathogenic isolates. *Aspergillus fumigatus* is able to grow at temperatures up to 50°C, which can be used in identification of that species (Sutton et al, 1998). Presumptive identification at the genus level is usually based on morphological characteristics, although species level identification particuallry of unusual species may be significantly more difficult. Cryptic species within each Aspergillus species complex pose a rela challenge in species identification which reauire polyphasic apparoaches (Samson et al, 2006). Specific identification of *Aspergillus* has become important, as differences in pathogenicity as well as susceptibility have been recognized. Key features of these three species most frequently associated with clinical disease are outlined below.

Aspergillus fumigatus (Figures 1.1-A) is the most common species to cause invasive infection, comprising more than 70% of the isolates identified in some series. The organism is found widespread in nature in soil, on decaying vegetation, in the air, and in water supplies (Anaissie et al, 2002). Its thermotolerance permits a wide range of diseases, which can also be used to identify the species. Colonies demonstrate a blue–green color with a wooly to cottony texture (Sutton et al, 1998). Hyphae are septate and hyaline with columnar conidial heads. Conidia are smooth-walled, uncolored and uniseriate with closely compacted phialides only on the upper portion of the vesicle. Conidia are smooth to finely roughened and are 2–3.5 μm in diameter. Hyphae, which are strongly angioinvasive, may not always branch at 45°C. Fruiting heads may occur rarely in clinical specimens in sites exposed to air (Sutton et al, 1998).

Aspergillus flavus (Figure 1.1-B) is found in soil and decaying vegetation. Colonies are olive to lime green and grow at a rapid rate. This species is typically biseriate with rough conidiophores and smooth conidia 3–6 μm that serve to distinguish the species. Some isolates are uniseriate. The organism is a common cause of sinusitis as well as invasive infection in immunosuppressed hosts. It is not only one of the most abundant and widely distributed soil-borne molds that can be found anywhere on earth but also produces aflatoxins, among the most carcinogenic natural products ever discovered (Jelinek et al., 1989; Denning, 1987). *A. flavus* is a saprobe capable of surviving on many organic nutrient sources like plant debris, tree leaves, decaying wood, animal fodder,

cotton, compost piles, dead insects and animal carcasses, stored grains, and even human and animal patients (Klich, 1998). Its optimal range for growth is between 25 - 37°C, but it can grow in a wide range of temperatures from 12 to 48°C. The ability of the fungus to grow at relatively high temperatures of the fungus contributes to its pathogenicity in humans and other warm blooded animals. For most of its life-cycle, stress from adverse conditions such as lack of adequate nutrients or water, causes the mycelia to form resistant structures called sclerotia. The fungus over-winters either as spores, as sclerotia or as mycelia in debris. Under favorable conditions sclerotia germinate directly to produce new colonies or conidiophores with conidia (Bennett et al., 1986; Cotty, 1988; Chang et al., 2002) Moreover, *A. flavus* is a weak and opportunistic plant pathogen, affecting many agricultural crops such as maize (com), cotton, groundnuts (peanuts), as well as tree-nuts such as Brazil nuts, pecans, pistachio nuts, almonds and walnuts. It can contaminate these crops with the secondary metabolite aflatoxin in the field before harvest. Aflatoxin has been named after Aspergillus flavus toxin. It also causes the spoilage of post harvest grains during storage (St Leger et al., 2000). Under weather conditions favorable for its growth, *A. flavus* can cause ear rot on maize, resulting in significant economic losses to farmers (Robens, 2001; Robens and Cardwell, 2005).

Aspergillus terreus (Figure 1.1-C) is common in tropical and subtropical habitats and has been increasingly reported as a cause of invasive infection in immunocompromised hosts (Iwen et al, 1998). Colonies are buff to beige to cinnamon (Sutton et al, 1998). Conidial heads are biseriate and columnar. Conidiophores are smooth walled and hyaline. Globose, sessile aleurioconidia are frequently produced on submerged hyphae. Conidia are small (2–2.5 µm). The colony color and fruiting structures are characteristic for this species, notable for its decreased susceptibility to some antifungal agents including amphotericin B (Iwen et al, 1998; Sutton et al, 1999).

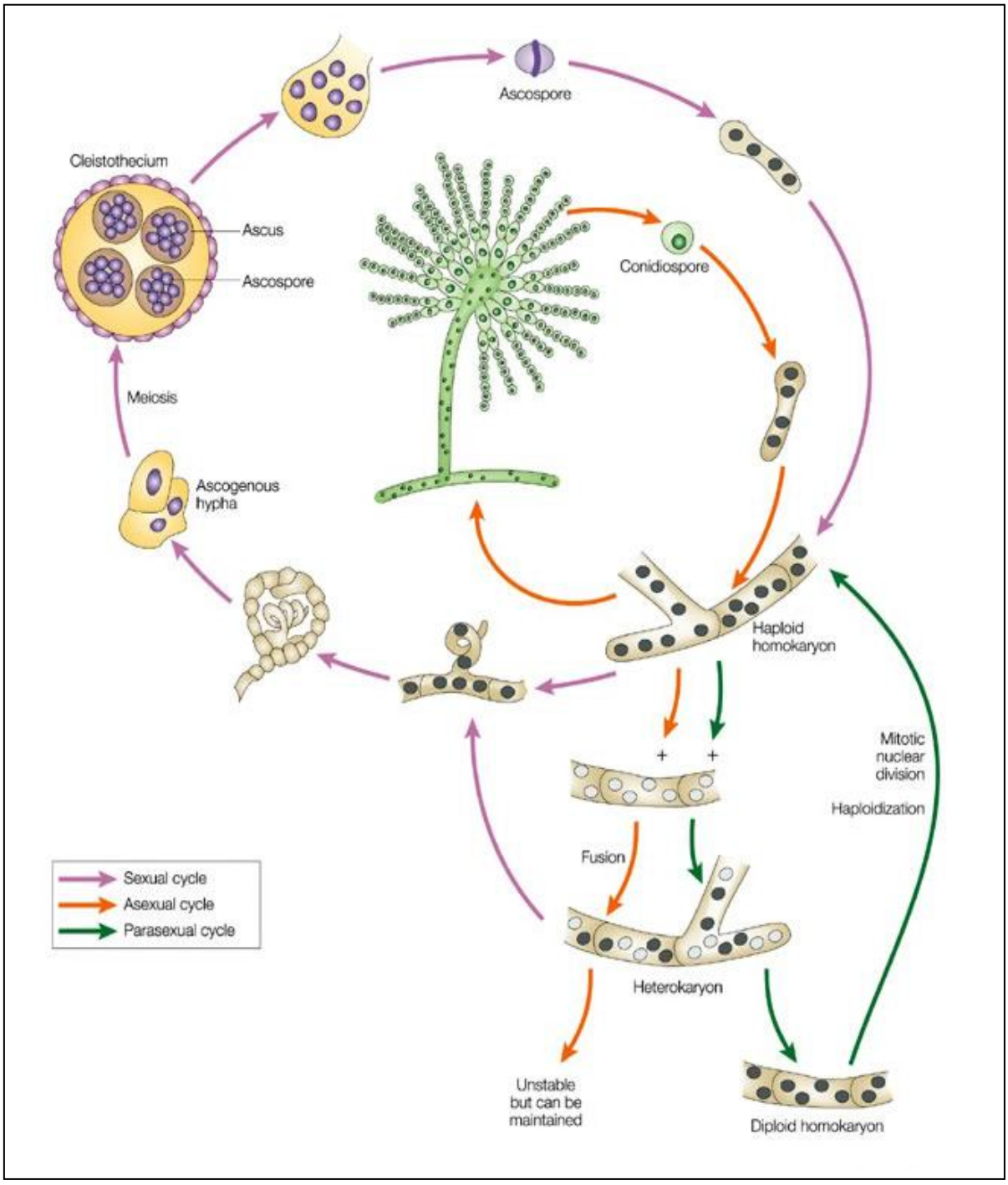

Figure 1.2. The parasexual, sexual and asexual (haploid) cycles present in the Aspergilli. The asexual cycle is the most common form of growth observed in Aspergillus species. The fungi grow across and through a substrate by hyphal elongation. When nutrients are exhausted, they conidiate and produce haploid spores that are then able to germinate and colonise new substrates.

1.3. Mycoses caused by Aspergillus

The clinical importance of *Aspergillus* pathogen has increased dramatically in recent decades. Invasive aspergillosis is a significant cause of morbidity and mortality in high risk

patients (Denning, 1998). A major factor associated with the increased number of cases of invasive aspergillosis is the increase in patients at risk for this disease, such as patients undergoing bone marrow or organ transplantation and patients with other immunodeficiencies. Unfortunately, definitive diagnosis of invasive aspergillosis remains difficult and occur frequently in the course of infection delaying the initiation of antifungal therapy (von Eiff et al, 1995; Stevens et al, 2000a). However, as rapid diagnostic tools like a PCR are not widely available to effectively establish a definite diagnosis of invasive aspergillosis, consideration of risk factors in specific epidemiological settings may be useful in suggesting a clinical diagnosis. In high-risk patients, clinical presentation as well as the use of diagnostic procedures such as radiology may be useful in establishing a presumptive diagnosis of infection (Caillot et al, 1997; Patterson, 1998; Stevens et al, 2000a). Cultures may not always be positive in patients with invasive aspergillosis, but it is important to recognize that a positive culture result in high-risk patients may suggest the presence of infection (Patterson, 1999b). However, even when therapy is begun promptly, therapeutic outcomes with current agents remain poor, particularly in patients with disseminated disease (Denning, 1998; Patterson et al, 2000b; Stevens et al, 2000a).

Several species of *Aspergillus* are opportunistic human pathogens that can cause a systemic disease named aspergillosis. In 1939, aspergillosis was described as "being so rare as to be of little practical importance" (reviewed in Latgé and Steinbach, 2009). Much has changed in the past 70 years. *Aspergillus fumigatus became* one of the most common fungal pathogens (Lass-Florl, 2009) and is regarded as the most important airborne pathogenic filamentous fungus in developed countries (Latgé, 2001). Other *Aspergillus* species can also cause human infections, particularly *A. flavus, A. terreus*, and *A. niger*, but also to a lesser extent *A. nidulans* (Mirhendi et al., 2007). The lungs are the most common site of *Aspergillus* infection, which can be acquired by inhaling conidia that reach the lungs to cause localized non-invasive infection called a pulmonary aspergilloma that can develop in a preexisting cavity (Thornton, 2010), Aspergillomas can also form in the brain, kidneys or other organs. *Aspergillus* species can produce allergic conditions such as allergic sinusitis (Walsh et al., 2008). Aspergillomas and allergic conditions are usually associated with low morbidity, and are seldom life-threatening (Riscili and Wood, 2009).

Invasive aspergillosis happens when *Aspergillus* invade tissues usually lung parenchyma whereas disseminated aspergillosis when the fungus reaches the blood and

spreads into other organs. Central nervous system aspergillosis has been reported in organ transplantation patients (Nadkarni, 2010). Despite aggressive treatment, invasive aspergillosis is still associated with high morbidity and mortality (Menzin et al., 2009).

Treatment of systemic aspergillosis is challenging because fungi share many metabolic pathways with mammals and therefore unique targets in fungi are limited. Most current antifungal drugs have limited efficacy and high toxicity. Mortality from aspergillosis exceeds 50% even with aggressive treatment.

1.3.1. Pathogenesis and Host Defenses

Aspergillus infection is typically acquired through inhalation of conidia into the lungs although other routes of exposure may also occur, such as oral or aerosol exposure to contaminated water (Anaissie and Costa, 2001) or though contact exposure, including surgical wounds, contaminated intravenous catheters or arm boards leading to cutaneous infection (Allo et al, 1987; Walsh, 1998; Gettleman et al, 1999). Invasive aspergillosis is uncommon in immunocompetent patients, although infection in apparently normal hosts does occur (Clancy and Nguyen, 1998; Patterson et al, 2000b). Hence, despite the ubiquitous nature of the organism and frequent exposure to it, normal host defenses do not readily permit invasive pulmonary aspergillosis to occur. *Aspergillus* species commonly produce toxins that may contribute to pathogenicity although they are unlikely to be major virulence determinants for systemic infection (Denning, 1987). These mycotoxins include aflatoxins, ochratoxin A, fumagillin, and gliotoxin. The latter particularly may compromise macrophage and neutrophil function (Denning, 2000). Aflatoxin, produced by *A. flavus*, is important as a carcinogenic and immunosuppressive agent but is not an important determinate of virulence of the organism (Denning, 1987). Other pathogenic features of the organisms include a variety of proteases and phospholipases (Latge, 1999), which contribute to pathogenicity. However, strains lacking the ability to produce these enzymes are still capable of causing experimental infection (Monod et al, 1993; Tang et al, 1993; Smith et al, 1994; Birch et al, 1996). The first lines of host defense against inhalation of *Aspergillus* conidia are ciliary clearance of the organism from the airways and the limited access to deep lung structures for larger, less pathogenic conidia. In the pulmonary tissues, the alveolar macrophage is a potent defense, capable of ingesting and killing inhaled *Aspergillus* conidia (Schaffner et al, 1982; Kan and Bennett, 1988). After

germination, the major line of defense against both swollen conidia and hyphae is the polymorphonuclear leukocyte. However, hyphae are too large to be effectively ingested and hyphal damage occurs extracellularly (Levitz et al, 1986). Swollen conidia and hyphae are both able to fix complement, which is important in phagocytic killing of the organism. Notably, *A. fumigatus* produces a complement inhibitor, which may play a role in its pathogenicity (Washburn et al, 1986; Washburn et al, 1987). Antibody responses due to prior exposures to *Aspergillus* are common but antibodies are not protective against invasive infection nor are they useful for diagnosis of infection, due to the fact that few immunosuppressed patients are able to mount an antibody response even in the setting of invasive disease (Young and Bennett, 1971).

The ability of phagocytes to oxidatively kill *Aspergillus* is a critical component of host defenses against the organism (Kan and Bennett, 1988). The use of corticosteroids substantially impairs neutrophil killing of hyphae and, which may be reversed to some degree with the use of interferon-γ or with administration of granulocyte or granulocyte-macrophage growth factors (Schaffner, 1985; Roilides et al, 1993). Recent studies have also suggested a role for cellular immunity. In a murine model of invasive aspergillosis, a Th1 response induced by administration of soluble interleukin-4 was associated with a favorable response (Cenci et al, 1997). In the clinical setting, prolonged neutropenia is a major risk factor for invasive aspergillosis (Gerson et al, 1984). Notably, response to even aggressive antifungal therapy is unlikely (Denning, 1998; Patterson et al, 2000b). Nevertheless, with changing patterns of immunosuppressive therapy, patients are less likely to remain neutropenic for extended periods of time and the use of growth factors has further shorten the duration of neutropenia. In recent surveys, other immunosuppressive conditions have emerged as important risk factors for invasive aspergillosis, and the time period for risk of invasive aspergillosis is now greatly extended. In patients receiving hematopoietic stem cell or marrow transplants, the period at risk now extends for more than 100 days after immunosuppression (Wald et al, 1997; Baddley et al, 2001), and reflects long-term complications of high-dose steroid therapy and other immunosuppressive agents for chronic graft-vs.-host disease that occurs following non myeloablative transplant procedures.

In noninvasive or allergic forms of aspergillosis, the pathogenesis is not well defined but appears to relate to chronic allergic responses to the organism (Stevens et al,

2000; Denning, 2001). The most frequent organism associated with allergic bronchopulmonary aspergillosis is *A. fumigatus*, with usual manifestations including central bronchiectasis, typical allergic features of bronchiolitis and chronic eosinophilic pneumonia (Rosenberg et al, 1977). Similarly, the pathogenesis of aspergilloma is not well defined but also seems to be associated with allergic reactions to chronic colonization. In aspergilloma, the organism does not usually invade the tissues but colonizes a pulmonary cavity. Although tissue invasion resulting in a chronic necrotizing form of aspergillosis can occur, the pathogenic features leading from colonization to invasive disease are not clearly understood (Tomlinson and Sahn, 1987).

1.3.2. Epidemiology

Aspergillus is a ubiquitous saprophytic mould that is found worldwide in a variety of habitats. *Aspergillus* is found in soil, water, food, and is particularly common in decaying vegetation. The inoculum for establishing infection is not known, but persons with normal pulmonary defense mechanisms can withstand even extensive exposure without any manifestation of disease, while severely immunocompromised hosts are susceptible to presumably lower inocula for establishing disease. Notably, patients may manifest infection due to prior asymptomatic colonization that leads to invasive infection when immunosuppressive conditions occur (such as neutropenia, corticosteroid use, or AIDS) (Walsh and Dixon, 1989).

Consideration of the epidemiology and host risk factors for aspergillosis may be useful in suggesting a clinical diagnosis of invasive infection. Neutropenia remains a major risk factor for invasive aspergillosis, but increasing numbers of patients with other risk factors, including solid organ and bone marrow transplantation, develop infection (Patterson et al, 2000; Stevens et al, 2000). In patients undergoing hematopoetic stem cell or marrow transplant, increased incidence of invasive aspergillosis has been reported (Marr et al, 2002). The epidemiology of invasive aspergillosis in those patients has shifted to the development of infection late in the course of transplantion with cases occurring more than 100 days after transplant due to risk factors of chronic graft-vs.-host disease, steroid use and other factors leading to prolonged immunosuppression (Wald et al, 1997; Marr et al, 2002). Recent series have shown that patients undergoing bone marrow

transplantation or receiving chemotherapy for hematological malignancies still constitute the majority of patients diagnosed with invasive aspergillosis (Patterson et al, 2000).

Among patients with solid organ transplants, those undergoing lung transplantation are at particular risk for *Aspergillus* infection with a clinical presentation ranging from an ulcerative tracheobronchitis to disseminated infection (Paterson and Singh, 1999; Patterson et al, 2000). Data from liver transplant recipients (1998–2001) indicate that invasive aspergillosis is more likely to involve the lung, occurs later in the post-transplantation period, and is associated with a lower mortality rate, compared to earlier liver transplant recipients (1990–1995) (Singh et al, 2003). Actually, the specific risk factors include not only neutropenia and graft-vs.-host disease, but also high dose corticosteroids and other immunosuppressive therapies. In addition, invasive aspergillosis has been observed among patients with advanced AIDS, especially in those with CD4 cell counts below 50/mm^3 (Denning et al, 1991). Standards for air quality in bone marrow transplant units, particularly spore filtration with high-efficiency particulate air (HEPA) filtration, have been recommended during the period of most severe neutropenia in order to reduce the rates of nosocomial infection (Patterson et al, 2000). Outbreaks of invasive aspergillosis have occurred in association with construction and other environmental risks.

Air filtration and infection control measures such as construction barriers that reduce risks by limiting exposure to aerosols have been shown to reduce the incidence of infection. However, in the most severely immunosuppressed patients, aspergillosis may still occur either as a result of endogenous reactivation of latent tissue infection or due to other exposures, perhaps even related inhalation of aerosols of contaminated water (Iwen et al, 1993; Patterson et al, 1997; Patterson et al, 2000; Anaissie and Costa, 2001). In addition, because bone marrow transplant and other organ transplant patients frequently receive substantial portions of their care in the outpatient setting, control of air quality in those settings is not possible and may result in exposure to *Aspergillus* conidia.

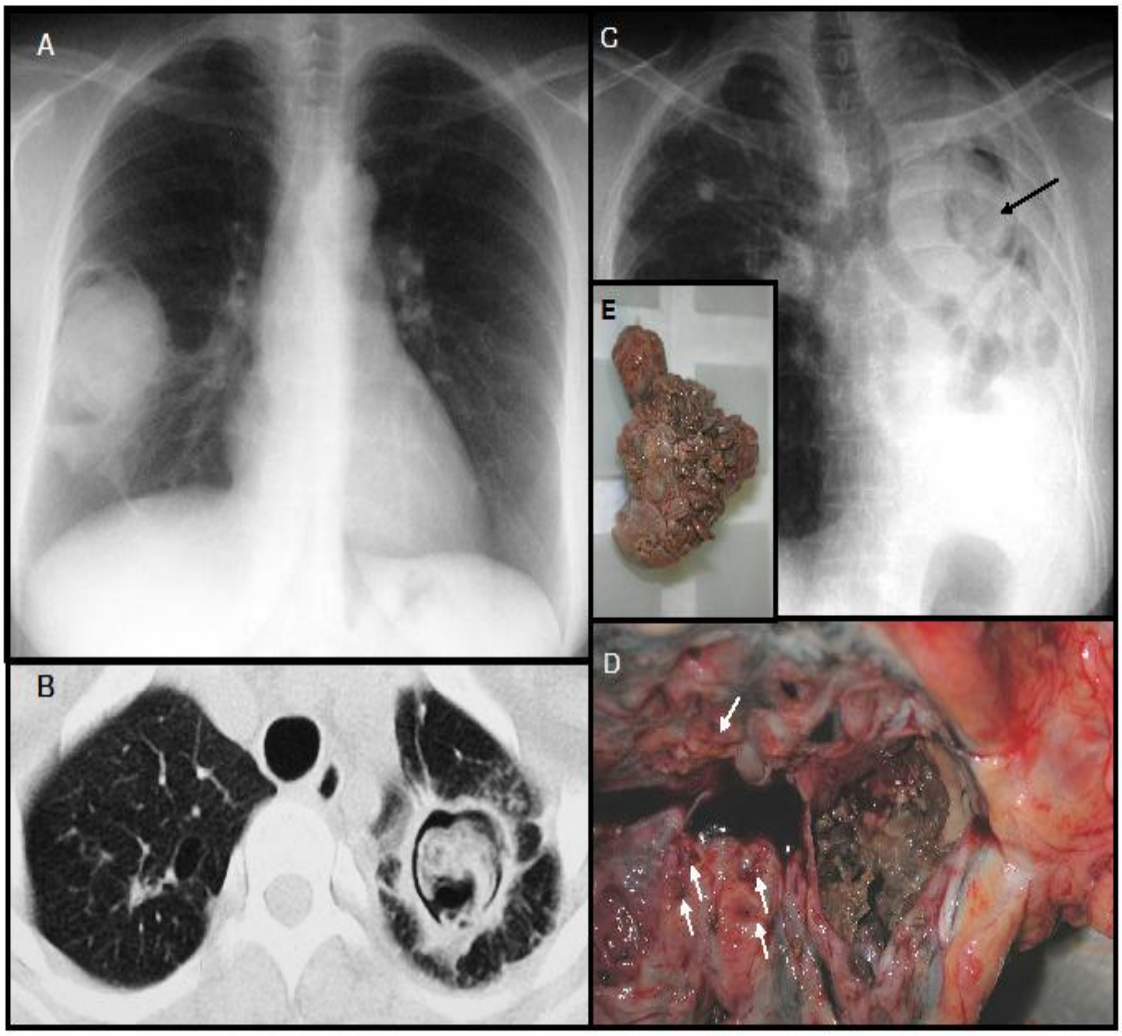

Figure 1.3. **A:** chest radiograph of patient, with a large right sided aspergilloma contained within a pre-existing, presumably congenital, bulla. **B**: thoracic computed tomography scan of both apices of the lung in another patient demonstrating a thick walled cavity and surrounding inflammatory reaction, and an irregular aspergilloma within the cavity. **C:** chest radiograph of third patient, with complete fibrotic destruction of the left lung which occurred over 5 years and a large aspergilloma visible in a large cavity in the upper mid zone (arrow) with multiple other empty cavities surrounding this. **D and E:** autopsy, there were numerous interconnected cavities in the mid and upper parts of the left upper lobe (white arrows), 2 containing aspergillomas.

1.3.3. CLINICAL SYNDROMES

1.3.3.1. Saprophytic Colonization and Superficial Aspergillosis

1.3.3.1.1. *Pulmonary aspergilloma*

A pulmonary fungus ball due to *Aspergillus* (aspergilloma) (figure 1.3) is characterized by chronic, extensive colonization of *Aspergillus* species in a pulmonary

cavity or ectatic bronchus. Fungus balls may also develop in other sites such as the maxillary or ethmoid sinus or even in the upper jaw following endodontic treatment (de Carpentier et al, 1994; Ferguson, 2000; Gillespie and O'Malley, 2000). Typically *Aspergillus* fungus balls in the lung develop in cavities resulting from pre-existing diseases or infections such as tuberculosis, histoplasmosis, sarcoidosis, bullous emphysema, fibrotic lung disease, or *Pneumocystis jirovecii* pneumonia in AIDS patients. *Aspergillus* is more common as the etiologic agent although other organisms such as *Scedosporium apiospermum* (*Pseudallescheria boydii*) or agents of zygomycosis can also cause similar syndromes.

The diagnosis of a pulmonary fungus ball is usually made radiographically; aspergilloma appears as a solid round mass inside a cavity. The detection of *Aspergillus* antibodies provide further evidence that the radiographic findings are consistent with a diagnosis of fungus ball due to *Aspergillus* and a biopsy is not usually undertaken (Rafferty et al, 1983). Although the presence of a fungus ball due to *Aspergillus* may be relatively asymptomatic, in some patients tissue invasion may occur, leading to invasive pulmonary aspergillosis or a subacute chronic necrotizing form of the disease (Tomlinson and Sahn, 1987). Hemoptysis is a common clinical symptom and can lead to a fatal complication (Aslam et al, 1971; Kauffman, 1996). Hemoptysis has been reported as the cause of death in up to 26% of patients with aspergilloma (Aslam et al, 1971; Kauffman, 1996). Management of aspergilloma is determined by the frequency and severity of hemoptysis as well as evaluation for risk factors that are associated with a poor prognosis. Complications are more likely in patients with severe underlying lung disease, immunosuppression or extensive disease, suggested by high titers of Aspergillus antibody. In these settings, specific therapies (corticosteroids and antifungal therapy) may be needed earlier in the course of management in order to attempt to avoid potential life-threatening hemoptysis (Kauffman, 1996).

1.3.3.1.2. *Other Superficial or Colonizing Conditions of Aspergillosis*

Aspergillus can also be associated with fungal balls of the sinuses without tissue invasion (Vennewald et al, 1999; Ferguson, 2000). The maxillary sinus is the most common site for a sinus aspergilloma (Ferguson, 2000). Clinical presentation is similar to that for any chronic sinusitis with chronic nasal discharge, sinus congestion and pain. The

diagnosis of a fungal ball may be suggested on computed tomography of the sinuses along with positive cultures for the organism, which is usually *A. fumigatus* or *A. flavus*.

Management is usually directed at surgical removal of the lesion with confirmation that the fungal ball has not caused bony erosion. Otomycosis is a condition of superficial colonization by *Aspergillus*, most typically *A. niger* (Kaur et al, 2000). The usual clinical presentation is that of an external otitis media, with ear pain and discharge. Examination of the ear canal may reveal the black conidiophores of *A. niger*. Treatment involves cleaning of the external ear canal and the topical administration of a variety of agents including cleansing solutions and topical antifungal agents. Other superficial or colonizing conditions due to *Aspergillus* include onychomycosis, which can be a chronic condition not responsive to antifungal agents directed at yeasts. Consequently, culture confirmation of *Aspergillus* as the etiologic agent will be useful in this setting. In addition, *Aspergillus* species may colonize the airways in patients with a variety of lung conditions that do not have apparent disease. A recent report by the Mycoses Study Group showed that a large number of clinical isolates of *Aspergillus* are not associated with infection (Perfect et al, 2001). Many of them were isolated from sputum samples of patients without apparent invasive disease, although the role of *Aspergillus* in causing symptoms of occasional hemoptysis and bronchitis in those patients is unclear.

1.3.3.2. Allergic Manifestations of Disease

1.3.3.2.1. *Allergic Bronchopulmonary Aspergillosis*

Allergic bronchopulmonary aspergillosis (ABPA) is a chronic allergic response to colonization with *Aspergillus*. Specific criteria for establishing a diagnosis include: (1) episodic bronchial obstruction (asthma); (2) peripheral eosinophilia; (3) immediate skin test reactivity to *Aspergillus* antigen; (4) precipitating *Aspergillus* antibodies; (5) elevated serum immunoglobulin E (IgE); (6) history of or presence of pulmonary infiltrates; and (7) central bronchiectasis (Rosenberg et al, 1977). The detection of the first six criteria establishes a likely diagnosis, while the presence of all seven confirms the condition.

Other secondary features that may be present include positive sputum cultures for Aspergillus, brown mucus plugs in sputa, elevated specific IgE antibodies against Aspergillus, late skin reactivity to Aspergillus, and reactions following intrabronchial challenge with Aspergillus (Patterson et al, 1982). In ABPA, typically the initiating event is

for an asthmatic patient to develop an allergic reaction to inhaled *Aspergillus*. Following that reaction, mucus plugs develop in the bronchi and can be detected by the presence of hyphae in sputa. The impacted mucus causes atelectasis, which in turn causes transient pulmonary infiltrates; repeated bronchial reactions ultimately lead to bronchiectasis in the proximal bronchi. Characteristic "ring signs" (circular or oblong densities) or "tram lines" (parallel shadows) are seen on radiographs.

These findings result from chronic peribronchial inflammation around dilated bronchi (Malo et al, 1977). Allergic bronchopulmonary aspergillosis is reported to occur in up to 14% of patients with steroiddependent asthma (Basich et al, 1981; Kumar and Gaur, 2000), and is also particularly common in patients colonized with *Aspergillus* such as those with cystic fibrosis who have a 7% prevalence of ABPA (Mroueh and Spock, 1994). In cystic fibrosis patients undergoing lung transplantation, the presence of *Aspergillus* colonization is an important risk for developing invasive pulmonary aspergillosis (Stevens et al, 2000). Allergic bronchopulmonary aspergillosis typically progresses through a series of stages which can be useful in approaching management of the condition: (1) acute; (2) remission; (3) exacerbation; (4) steroiddependent asthma; and (5) fibrosis (Patterson et al, 1982). The initial acute stage is usually responsive to corticosteroid therapy which may lead to a period of asymptomatic remission. Most patients will experience exacerbations and may eventually become steroid dependent. Late stage manifestations include pulmonary fibrosis that may be associated with substantially reduced pulmonary function and is associated with a poor long-term prognosis. Management of ABPA is directed at reducing acute asthmatic symptoms and avoiding end-stage fibrotic complications. Corticosteroid therapy is commonly used for treating exacerbations although few randomized trials have been conducted to evaluate its use (Stevens et al, 2000). However, increasing serum IgE levels, worsening or new infiltrates, or worsening findings on spirometry suggest that steroids may be helpful (Stevens et al, 2000). The role of antifungal agents is limited although itraconazole may be beneficial in reducing symptoms and reducing the use of steroids.

1.3.3.2.2. *Other Allergic Manifestations*

Allergic responses can also contribute to symptoms of sinusitis (Katzensteinm et al, 1983; Corey et al, 1995; De Shazo et al, 1997). Allergic sinusitis is similar in its

presentation to sinusitis complicated with fungal balls due to *Aspergillus* (Houser and Corey, 2000). Frequently, in patients with allergic fungal sinusitis, polyposis or eosinophil-rich mucin containing Charcot-Leyden crystals may be seen (Washburn, 1998). Management is largely directed at confirming lack of invasive infection and in aerating the sinus (Kuhn and Javer, 2000). The use of steroids or antifungal agents has not been conclusively demonstrated to be of benefit (Kuhn and Javer, 2000).

1.3.3.3. Invasive Syndromes Caused by Aspergillus

Invasive Pulmonary Aspergillosis (IPA) is the most common manifestation of invasive aspergillosis. Infection with *Aspergillus* is usually acquired through inhalation of airborne conidia, which invade the lung tissue in the absence of an effective monocytic or neutrophilic immune response (Latge, 2001). Hyphal invasion into blood vessels is common, occurring in approximately a third of patients with IPA. Nonpulmonary sites become infected by contiguous spread or via hematogenous spread to the central nervous system (occurring in 10 to 40% of severely immunosuppressed patients such as those undergoing allogeneic stem cell or bone marrow transplant) or to other organs including the liver, spleen, kidney, skin, bone, and heart (Denning et al, 1998; Latge, 1999; Ribaud et al, 1999).

Response to antifungal therapy depends on several factors including the immune status of the host and extent of the infection at time of diagnosis. The complete resolution of signs and symptoms attributed to invasive aspergillosis, and partial responses (substantial improvement), are seen in less than 40% of treated patients. Even more striking are the extremely poor responses seen in the most highly immunosuppressed patients. For example, in a survey of 595 patients with invasive aspergillosis, favorable responses were seen in only 13% of patients undergoing allogeneic bone marrow transplantation (Patterson et al, 2000). Similarly poor responses have been reported in other recent series as well, although newer therapeutic modalities like the triazoles may improve the outcome even in patients with more severe immunosuppression (Maertens et al, 2000; Bowden et al, 2002; Herbrecht et al, 2002). Extent of infection also correlates with likelihood of a favorable outcome: in patients with disseminated aspergillosis favorable responses decrease to less than 20% and to less than 10% in patients with

central nervous system disease. As might be predicted, the mortality rate of patients with invasive aspergillosis also correlates with immune status of the host and extent of disease.

1.4. Diagnosis

The diagnosis of invasive aspergillosis continues to be made largely on clinical grounds and histopathological findings, although extensive efforts are ongoing to establish a panel of tests and procedures to facilitate more rapid diagnosis. An important clue for the diagnosis of IPA, for example, is the presence of signs and symptoms that result from angioinvasion of pulmonary veins and arteries by the fungus. This process produces clinical features that may include acute dyspnea, pleuritic chest pain, or hemoptysis. The diagnosis of invasive aspergillosis is proven with demonstration of tissue hyphal invasion together with a positive culture for Aspergillus species (Ascioglu et al, 2002). However, tissue samples are often reluctantly undertaken because of the severe thrombocytopenia that is frequently present in patients at high risk for the disease. Hyphal elements and morphology are usually easily demonstrated using fungal stains, such as Gomori methenamine silver or periodic acid-Schiff. In acute invasive aspergillosis, the hyphae are typically hyaline, septate and 3–6 μm in width with parallel cross-walls and are dichotomously branched at acute angles (Sutton et al, 1998). These features usually allow distinction from Zygomycetes, which are much broader, exhibit right angle branching, and are rarely septate. In addition, agents of phaeohyphomycosis can be distinguished due to their black or darkly pigmented hyphae with specific melanin staining with the Fontana-Masson stain (Dixon and Polak-Wyss, 1991). However, it is important to note that a number of pathogenic moulds, including Scedosporium, Fusarium, Geotrichium, Scopulariopsis and others will have virtually identical appearances on histopathology regardless of the stains used. Although specific immunohistochemical stains using fluorescent antibodies to *Aspergillus* can distinguish the organism, they are widely available (de Repentigny et al, 1994; Kaufman et al, 1997). Genus and species identification and susceptibility testing are becoming more important as antifungal therapies are increasingly directed at specific pathogens. For example, the newer triazoles, such as voriconazole, are extremely active against *Aspergillus* but variably active against Zygomycetes (Espinel-Ingroff et al, 2001; Pfaller et al, 2002). Similarly, the echinocandins are not active against Zygomycetes and have limited activity against moulds other than

Aspergillus. Additionally, identification of the organism will also allow susceptibility testing to be performed. Recently, susceptibility testing for moulds including *Aspergillus* has been validated (Espinel-Ingroff et al, 1997; Espinel-Ingroff et al, 2000), but correlation with clinical results has not yet been established. Antifungal resistance to itraconazole has been reported for a limited number of isolates, which correlates with lack of efficacy in an animal model. In addition, these isolates may not be cross-resistant to other azoles in vitro (Denning et al, 1997; Mosquera and Denning, 2002). In addition, *A. terreus* may be resistant to amphotericin B and susceptible to azoles so that testing of that species could also be of potential clinical utility (Sutton et al, 1998).

A number of approaches can be utilized to obtain tissue samples in cases of suspected invasive aspergillosis. For pulmonary lesions and other sites, such as deep organs or bone, a fine needle biopsy can be attempted and guided by CT or fluoroscopy to increase the diagnostic yield and avoid a more invasive procedure. Bronchoscopies with bronchoalveolar lavage and transbronchial biopsies are useful in establishing a specific diagnosis and in evaluating for multiple pathogens in these high-risk patients (Albelda et al, 1984; Kahn et al, 1986). Brain lesions that are not accessible can be presumptively identified with the presence of invasive pulmonary aspergillosis and/or disseminated infection diagnosed elsewhere (Perea and Patterson, 2002).

1.4.1. Culturing

Cultures for *Aspergillus* in respiratory samples may be associated with infection. Recent studies have demonstrated in high-risk patients with increased rates of infection, such as patients with neutropenia or those undergoing bone marrow transplantation, that the presence of *Aspergillus* in a respiratory sample, particularly if obtained via bronchoalveolar lavage, is highly suggestive of the diagnosis of invasive aspergillosis (Yu et al, 1986; Horvath and Dummer, 1996). By contrast, blood cultures are rarely positive for *Aspergillus*. These features have led to standardized diagnostic criteria developed by a joint Mycoses Study Group/European Organization for Research and Treatment of Cancer (MSG/EORTC) panel. Accordingly, the positive respiratory cultures in conjunction with clinical illness and pulmonary infiltrates in neutropenic or bone marrow transplant patients constitute criteria for a probable diagnosis of invasive pulmonary aspergillosis (Ascioglu et al, 2002).

1.4.2. Radiographic Diagnosis

Radiographic findings can also be helpful in establishing a diagnosis of *Aspergillus* infection. Plain chest radiographs are insensitive as extensive pulmonary disease may be present with few findings on chest films. In neutropenic and bone marrow transplant patients with invasive aspergillosis and other angioinvasive moulds, chest computed tomography (CT) scans may demonstrate lesions that are not visible on plain radiographs. A "halo" of low attenuation surrounding a nodular lung lesion in a high risk patient has been associated with an early diagnosis of infection (Caillot et al, 1997). A nodular lesion may undergo cavitation and subsequently be associated with an "air crescent" sign that is associated with aspergillosis; this radiographic sign occurs later in the course of illness, usually after recovery of neutrophils (figure 1.4) (Caillot et al, 1997).

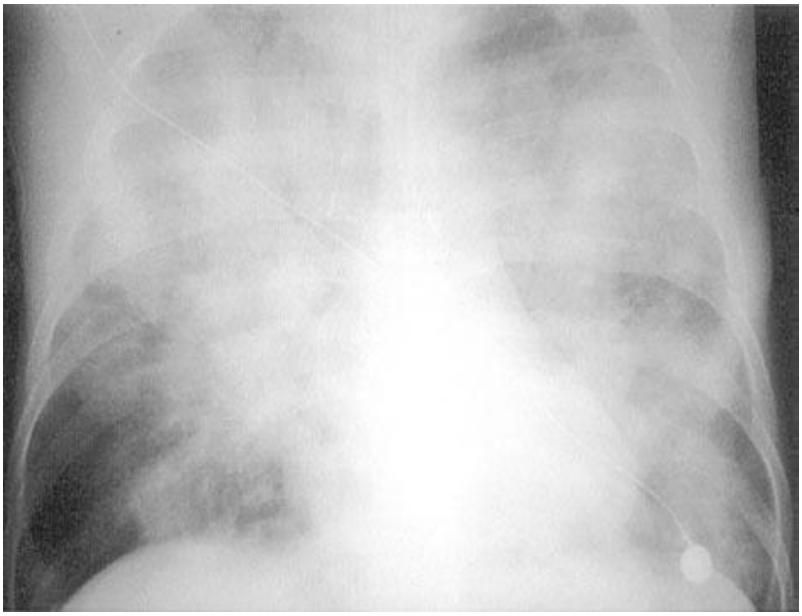

Figure 1.4. Chest radiograph showing diffuse nodular infiltrates in a patient with invasive pulmonary aspergillosis

Recent studies have demonstrated that the presence of a CT "halo" sign as a trigger to begin presumptive therapy for IPA in high-risk patients resulted in very favorable response rates particularly when combined with surgical resection of isolated lesions (figure 1.5) (Yeghen et al, 2000).

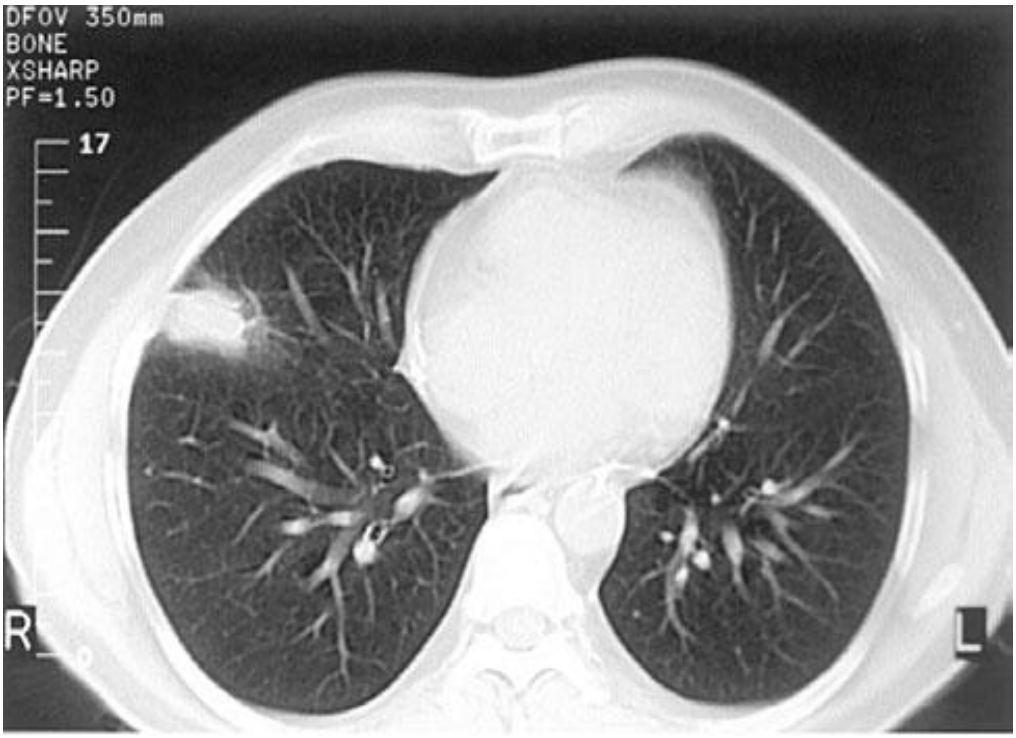

Figure 1.5. Computed tomography of chest showing "halo" sign of low attenuation surrounding a nodular lung lesion in patient with early invasive pulmonary aspergillosis

1.4.3. Galactomannan ELISA assay

The utility of non-culture methods to establish a rapid diagnosis of invasive aspergillosis has been vigorously investigated (Verweij et al, 1998). Antibody detection was initially evaluated, but a large proportion of patients have antibodies due to prior asymptomatic exposure to ubiquitous *Aspergillus* conidia. Even more problematic for utilizing antibody for diagnosis of invasive disease is the fact that highly immunosuppressed patients will not develop a prompt antibody response to infection. A number of investigators have evaluated various methods to establish a diagnosis of invasive infection by detecting *Aspergillus* antigens or metabolites in serum, but the clinical utility of these methods remain limited, due in part to the lack of widespread availability of these tests (Patterson et al, 1997; Verweij et al, 1998).

A sandwich enzyme-linked immunoassay ELISA that utilizes a monoclonal antibody to galactomannan antigen has been developed(Platelia Aspergillus EIA, Bio-Rad Laboratories, UK). It is considered one of the most important diagnostic advances in recent years and has been included among the mycological criteria for probable invasive fungal disease by EORT/MSG Consensus Group (De Pauw B et al, 2008). The galactomannan ELISA assay has been used most extensively in Europe where it is

approved for clinical use (Verweij et al, 1995). This system uses the monoclonal antibody EB-A2 that is also used in the Pastorex Aspergillus latex agglutination test (Sanofi Diagnostics Pasteur), which has limited sensitivity for the detection of circulating antigen. However, in contrast to the latex agglutination test, the sandwich ELISA lowers the detection limit for antigen 10-fold, to 0.5–1.0 ng/ml of galactomannan in serum by using the antibody both as a captor and as a detector (Verweij et al, 1995). Serial testing of serum specimens is advised for obtaining early diagnosis and increased specificity. Test sensitivity and specificity corresponded to 71% and 89%, respectively, in a meta-analysis. The assay appears to be also diagnostically useful for testing bronchoalaveolar lavage or CSF specimens (Cuenca-Estrella et al, 2011).

With this ELISA assay, sensitivity for detecting invasive aspergillosis is more than 90% with a positive predictive value of more than 80% (Maertens et al, 2001).

1.4.4. PCR Detection

The use of PCR to detect invasive fungal pathogens, including *Aspergillus*, has been reported but its application is still limited to reference laboratories applying mostly in-house protocols. False positive results due to the ubiquitous nature of fungal conidia or contamination have thus far limited its clinical utility (Hebart et al, 2000; Loeffler et al, 2001). Low sensitivity is also an important issue, while the optimal clinical specimen has not yet been defined. International collaborative efforts are currently underway to standardize testing procedures, while the recent availability of at least two commercial amplification methods is expected to improve our knowledge about test utility (Cuenca-Estrella et al, 2011).

1.5. Clinical Pharmacology

It is the study of drug in human which has been termed a bridging discipline because it combines elements of classical pharmacology with clinical medicine. It includes both evalution of safety and efficacy of currently available drugs and development of new and improved pharmacotherapy (Reidenberg, 1999).

1.5.1. Pharmacokinetics

It is a quantitative analysis of the process of drug absorption, distribution and elimination that determine the time course of drug action. It is important tool that is used in the conduct basic applied research. Drugs injected intravenously are removed from the plasma through two primary mechanisms: (1) Distribution to body tissues and (2) metabolism and excretion of the drugs. The resulting decrease of the drug's plasma concentration follows a biphasic pattern (figure 1.6). **Alpha phase:** An initial phase of rapid decrease in plasma concentration. The decrease is primarily attributed to drug distribution from the central compartment (circulation) into the peripheral compartments (body tissues). This phase ends when pseudo-equilibrium of drug concentration is established between the central and peripheral compartments. **Beta phase:** A phase of gradual decrease in plasma concentration after the alpha phase. The decrease is primarily attributed to drug metabolism and excretion (Gill et al, 1999). **Additional phases** (gamma, delta, etc.) are sometimes seen (Weiner and Johan, 2000).

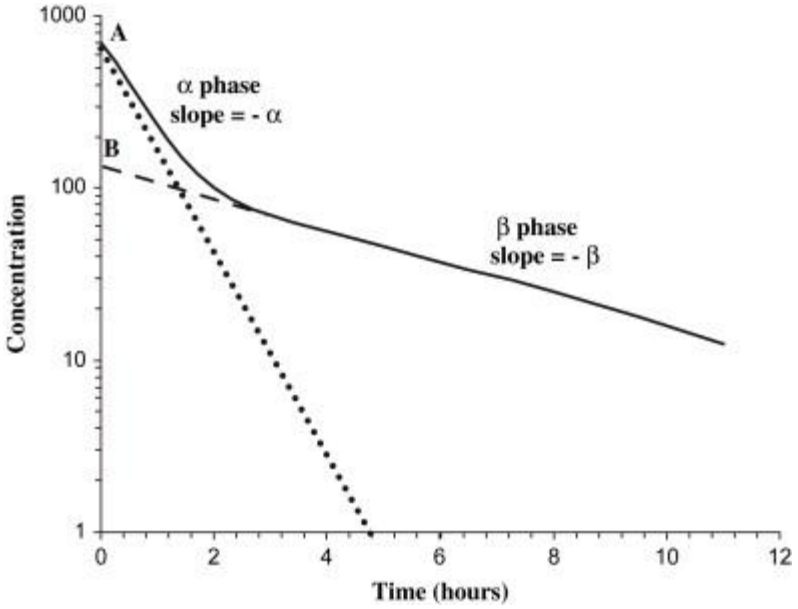

Figure 1.6. Plasma drug concentration vs time after dose adminstration.

1.5.1.1. Pharmacokinetic analysis

Pharmacokinetic analysis is performed by noncompartmental or compartmental methods. Noncompartmental methods estimate the exposure to a drug by estimating the area under the curve of a concentration-time graph. Compartmental methods estimate the concentration-time graph using kinetic models. Noncompartmental methods are often more versatile in that they do not assume any specific compartmental model and produce accurate results also acceptable for bioequivalence studies (Gill et al, 1999).

1.5.1.2.1. Noncompartmental analysis

Noncompartmental Pharmacokinetic analysis is highly dependent on estimation of total drug exposure. Total drug exposure is most often estimated by area under the curve (AUC) methods, with the trapezoidal rule (numerical integration) the most common method. Due to the dependence on the length of in the trapezoidal rule, the area estimation is highly dependent on the blood/plasma sampling schedule. That is, the closer time points are, the closer the trapezoids reflect the actual shape of the concentration-time curve (Clark, 1986).

1.5.1.1.1. Compartmental analysis

Compartmental Pharmacokinetic analysis uses kinetic models to describe and predict the concentration-time curve. Pharmacokinetic compartmental models are often similar to kinetic models used in other scientific disciplines such as chemical kinetics and thermodynamics. The advantage of compartmental over some noncompartmental analyses is the ability to predict the concentration at any time. The disadvantage is the difficulty in developing and validating the proper model. Compartment-free modeling based on curve stripping does not suffer this limitation. The simplest Pharmacokinetic compartmental model is the one-compartmental Pharmacokinetic model with IV bolus administration and first-order elimination. The most complex Pharmacokinetic models (called PBPK models) rely on the use of physiological information to ease development and validation (Evans et al, 1992).

1.5.1.2. Pharmacokinetic models

Pharmacokinetic models are hypothetical structures that are used to describe the fate of a drug in a biological system following its administration.

1.5.1.2.1. One-compartment model

Following drug administration, the body is depicted as a kinetically homogeneous unit. This assumes that the drug achieves instantaneous distribution throughout the body and that the drug equilibrates instantaneously between tissues. Thus the drug concentration–time profile shows a monophasic response (i.e. it is monoexponential) (figure 1.7).

It is important to note that this does not imply that the drug concentration in plasma (Cp) is equal to the drug concentration in the tissues. However, changes in the plasma concentration quantitatively reflect changes in the tissues. The relationship can be

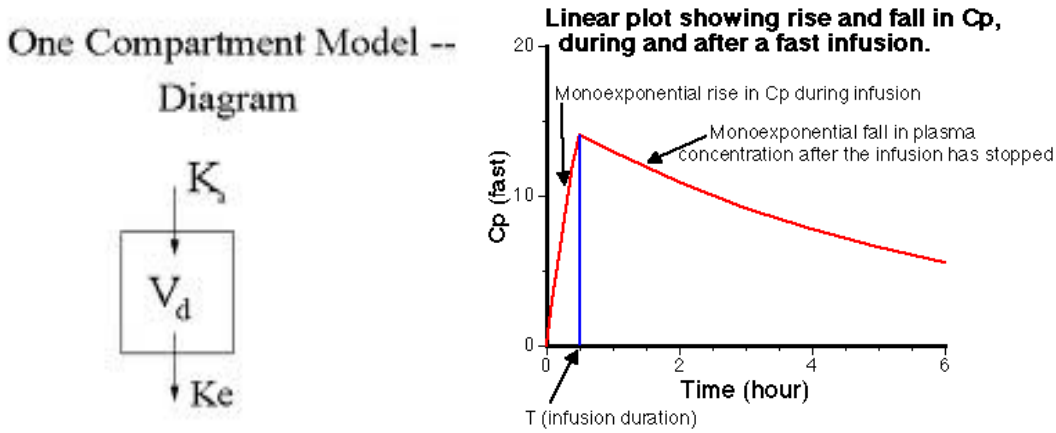

Figure 1.7. One-compartment pharmacokinetic model

plotted on a log Cp vs time graph and will then show a linear relation; this represents a one-compartment model (Evans et al, 1992).

1.5.1.2.2. Two-compartment model

The two-compartment model resolves the body into a central compartment and a peripheral compartment (figure 1.8). Although these compartments have no physiological or anatomical meaning, it is assumed that the central compartment comprises tissues that are highly perfused such as heart, lungs, kidneys, liver and brain. The peripheral compartment comprises less well-perfused tissues such as muscle, fat and skin.

A two-compartment model assumes that, following drug administration into the central compartment, the drug distributes between that compartment and the peripheral compartment. However, the drug does not achieve instantaneous distribution, i.e. equilibration, between the two compartments.

The drug concentration–time profile shows a curve, but the log drug concentration–time plot shows a biphasic responseand can be used to distinguish whether a drug shows a one- or two-compartment model. Figure 1.8 shows a profile in which initially there is a rapid decline in the drug concentration owing to distribution from the central compartment to the peripheral compartment (distribution phase). Hence during this rapid initial phase the drug concentration will decline rapidly from the central compartment, rise to a maximum in the peripheral compartment, and then decline.

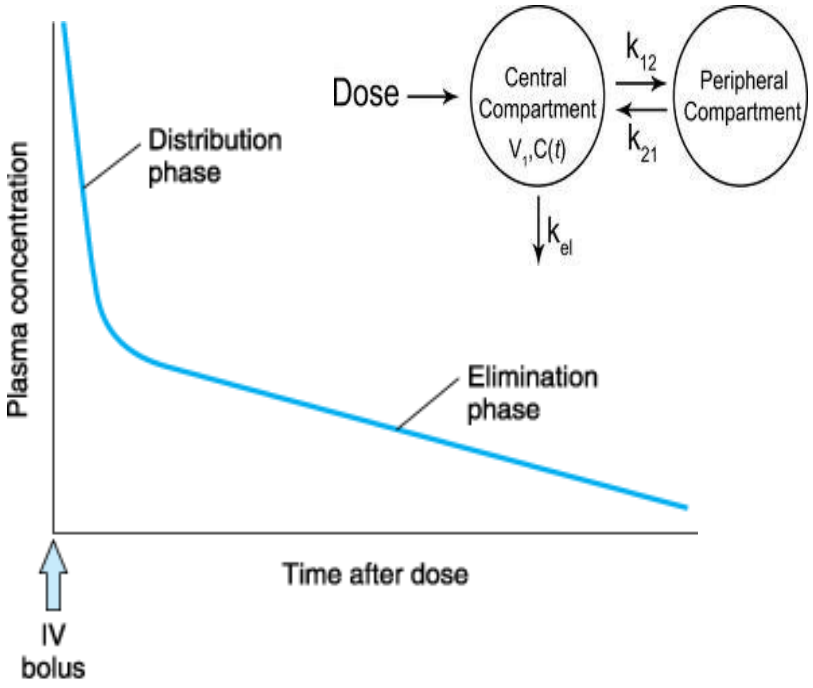

Figure 1.8. Two -compartment model of pharmacokinetic.

After a time interval (t), distribution equilibrium is achieved between the central and peripheral compartments, and elimination of the drug is assumed to occur from the central compartment (elimination phase). As with the onecompartment model, all the rate processes are described by first-order reactions (Evans et al, 1992).

1.5.1.2.3. Multi-compartment model

In this model the drug distributes into more than one compartment and the concentration–time profile shows more than one exponential. Each exponential on the concentration–time profile describes a compartment (Figure 1.9). For example, amphotericin B can be described by a three-compartment model following a single IV dose (Bekersky et al, 1992)

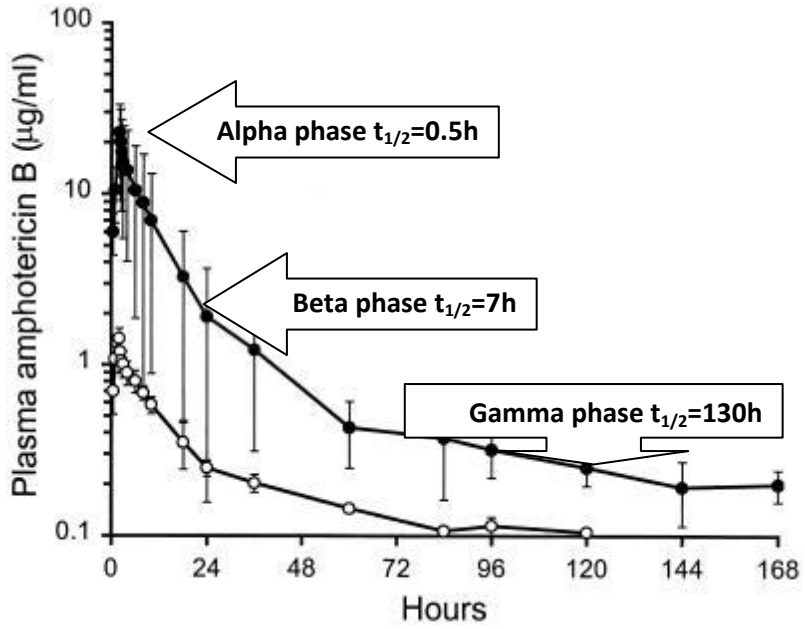

Figure 1.9. Plasma-cocnentration profile of liposomal amphotericin B (2 mg/kg, closed circles) and amphotericin B deoxycholate (0.6 mg of /kg (open circles) after a 2-h infusion of or in healthy subjects.

1.5.1.3. Pharmacokinetic parameters

This section describes various applications using the one-compartment open model system.

1.5.1.3.1. Elimination rate constant

Consider a single IV bolus injection of drug X. As time proceeds, the amount of drug in the body is eliminated. Thus the rate of elimination can be described (assuming first-order elimination) as: $dX / dt = Kx.$ Hence, $X = X_0 \, exp(-kt)$ where X: amount of drug X, X_0: dose and k: first-order elimination rate constant.

1.5.1.3.2. Volume of distribution

The volume of distribution (*Vd*) has no direct physiological meaning; it is not a 'real' volume and is usually referred to as the apparent volume of distribution. It is defined as that volume of plasma in which the total amount of drug in the body would be required to be dissolved in order to reflect the drug concentration attained in plasma.

The body is not a homogeneous unit, even though a one-compartment model can be used to describe the plasma concentration–time profile of a number of drugs. It is important to realise that the concentration of the drug (*Cp*) in plasma is not necessarily the same in the liver, kidneys or other tissues.

Thus *Cp* in plasma does not equal *Cp* or amount of drug (*X*) in the kidney or *Cp* or amount of drug (*X*) in the liver or *Cp* or amount of drug (*X*) in tissues. However, changes in the drug concentration in plasma (*Cp*) are proportional to changes in the amount of drug (*X*) in the tissues. Since Cp (plasma) ↔ Cp (tissues) i.e. Cp (plasma) ↔ X (tissues) Then *Cp* (plasma) = *Vd* x *X* (tissues) where Vd is the constant of proportionality and is referred to as the volume of distribution, which thus relates the total amount of drug in the body at any time to the corresponding plasma concentration. Thus, $V_d = X/C_p$ and *V*d can be used to convert drug amount *X* to concentration. Since $X = X_0 \exp(-kt)$, Then $X/V\text{d} = X_0 - \exp(kt)/V\text{d}$. Thus, $C_{pt} = C_p^0 \exp(-kt)$. This formula describes a monoexponential decay, where C_{pt} = plasma concentration at any time *t*. The curve can be converted to a linear form using natural logarithms (ln): $\ln C_{pt} = \ln C_{p0} - kt$, where the slope = *k*, the elimination rate constant; and the *y* intercept = ln C_{p0}. Since $V_d = X/C_p$ then at zero concentration (C_p^0), the amount administered is the dose, *D*, so that $C_p^0 = D/V_d$

If the drug has a large V_d that does not equate to a real volume, e.g. total plasma volume, this suggests that the drug is highly distributed in tissues. On the other hand, if the V_d is similar to the total plasma volume this will suggest that the total amount of drug is poorly distributed and is mainly in the plasma (Shargel et al, 2005).

1.5.1.3.3. Half-life

The time required to reduce the plasma concentration to one half its initial value is defined as the *half-life* ($t_{1/2}$). Consider $\ln C_{pt} = \ln C_p - kt$ and $t_{1/2} = 0.693/k$. This parameter is very useful for estimating how long it will take for levels to be reduced by half the original concentration. It can be used to estimate for how long a drug should be stopped if a patient has toxic drug levels, assuming the drug shows linear one-compartment pharmacokinetics.

1.5.1.3.4. Clearance

Drug clearance (CL) is defined as the volume of plasma in the vascular compartment cleared of drug per unit time by the processes of metabolism and excretion. Clearance for a drug is constant if the drug is eliminated by first-order kinetics. Drug can be cleared by renal excretion or by metabolism or both. With respect to the kidney and liver, etc., clearances are additive that is: $CL_{total} = CL_{renal} + CL_{nonrenal}$. Mathematically,

clearance is the product of the first-order elimination rate constant (*k*) and the apparent volume of distribution (*V*d). Thus, $CL_{total} = k \times V_d$. Hence the clearance is the elimination rate constant – i.e. the fractional rate of drug loss – from the volume of distribution.

Clearance is related to half-life by $t_{1/2=}0.693 \times V_d / CL$. If a drug has a CL of 2L/h, this tells you that 2 litres of the V_d is cleared of drug per hour. If the C_p is 10 mg/L, then 20 mg of drug is cleared per hour (Gibaldi et al, 1983).

1.5.1.4. Pharmacokinetic applications

Some drugs may be used clinically on a single-dose basis, although most drugs are administered continually over a period of time. When a drug is administered at a regular dosing interval (orally or IV), the drug accumulates in the body and the serum concentration will rise until steady-state conditions have been reached , assuming the drug is administered again before all of the previous dose has been eliminated.

In practice the glossary of equations described can be used to simulate plasma concentration vs time profiles for a dosage regimen using different routes of administration. The important issue is to utilize mean pharmacokinetics parameters derived from research that match the clinical and demographic data of the patient. Pharmacokinetic interpretation and estimation of a patient's actual pharmacokinetic data, e.g. Clearance, relies on plasma concentrations measured at a specific time following drug administration where this depicts the average plasma concentration.

Some drugs show nonlinear drug handing. The process of metabolism is nonlinear and the rate of metabolism shows zero order. In practice, Michaelis–Menten pharmacokinetics is applied the profile of the rate of metabolism of drug given at different dosages. The rate of elimination increases until it reaches a plateau where the rate of elimination is constant despite increases in the total daily dose of the drug. The profile can be described that the model appears to fit the pattern for the metabolic elimination of drug is not linear always and is proposed as the velocity (*V*) or rate at which an enzyme can metabolise a substrate (*C*p) can be described by the following equation: $V = Vm \times Cp / Km \times Cp$ where *V* is the rate of metabolism, *V*m (sometimes referred to as *V*max) is the maximum rate of metabolism and *K*m is the substrate concentration (*C*p) at which *V* will be half *V*m, i.e. when half the total enzyme is complexed with the substrate (Taylor and Diers-Caviness, 2003).

1.5.2. Pharmacodynamics

It refers to the relationship between drug concentration at the site of action and the resulting effect, including the time course and intensity of therapeutic and adverse effects. The effect of a drug present at the site of action is determined by that drug's binding with a receptor. Receptors may be within bacteria to disrupt maintenance of the bacterial cell wall.

For most drugs, the concentration at the site of the receptor determines the intensity of a drug's effect. However, other factors affect drug response as well. Density of receptors on the cell surface, the mechanism by which a signal is transmitted into the cell by second messengers (substances within the cell), or regulatory factors that control gene translation and protein production may influence drug effect. This multilevel regulation results in variation of sensitivity to drug effect from one individual to another and also determines enhancement of or tolerance to drug effects.In the simplest examples of drug effect, there is a relationship between the concentration of drug at the receptor site and the pharmacologic effect. If enough concentrations are tested, a maximum effect (E_{max}) can be determined (figure 1.9). When the logarithm of concentration is plotted versus effect, one can see that there is a concentration below which no effect is observed and a concentration above which no greater effect is achieved.

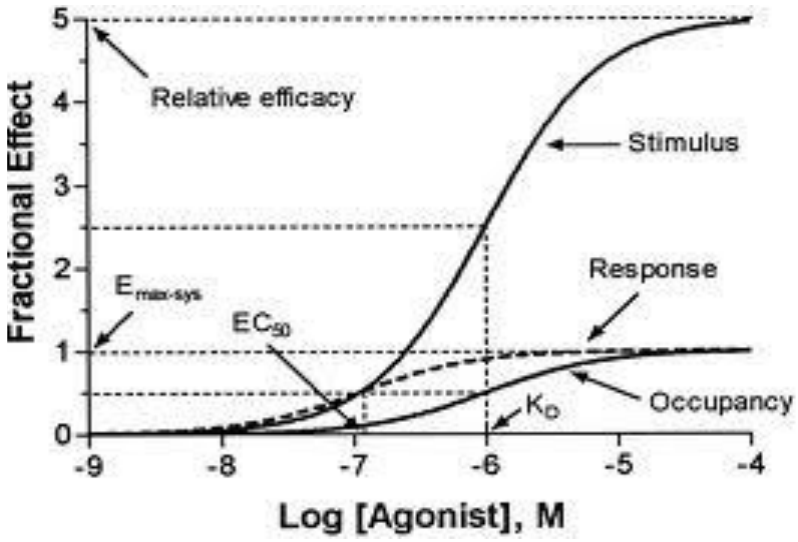

Figure 1.10. Relationship of drug concentration at the receptor site toeffect (as a percentage of maximal effect).

For some drugs, the effectiveness can decrease with continued use. This is referred to as *tolerance.* Tolerance may be caused by pharmacokinetic factors, such as increased drug metabolism, that decrease the concentrations achieved with a given dose. There can also be pharmacodynamic tolerance, which occurs when the same concentration at the

receptor site results in a reduced effect with repeated exposure. Tolerance can be described in terms of the dose– response curve, as shown in figure 1.10.

1.5.2.1. Pharmacodynamic Models

1.5.2.1.1. The E_{max} and Sigmoid E_{max} (non-linear) Models

The mathematical model that comes from the classic drug receptor theory shown previously is known as the E_{max} model:

$$E = \frac{E_{max} \times C}{EC_{50} + C}$$

where E is the pharmacologic effect elicited by the drug, E_{max} is the maximum effect the drug can cause, EC_{50} is the concentration causing one-half the maximum drug effect ($E_{max}/2$), and C is the concentration of drug at the receptor site. EC_{50} can be used as a measure of drug potency (a lower EC_{50}, indicating a more potent drug), whereas E_{max} reflects the intrinsic efficacy of the drug (a higher E_{max}, indicating greater efficacy. Duration of effect is determined by a complex set of factors, including the time that a drug is engaged on the receptor as well as intracellular signaling and gene regulation.

When dealing with human studies in which a drug is administered to a patient, and pharmacologic effect is measured, it is very difficult to determine the concentration of the drug at the receptor site. Because of this, serum concentrations (total or unbound) usually are used as the concentration parameter in the Emax equation. Therefore, the values of E_{max} and EC_{50} are much different than if the drug were added to an isolated tissue contained in a laboratory beaker.

The result is that a much more empirical approach is used to describe the relationship between concentration and effect in clinical pharmacology studies. After a pharmacodynamic experiment has been conducted, concentration–effect plots are generated. The shape of the concentration–effect curve is used to determine which pharmacodynamic model will be used to describe the data. Sometimes a hyperbolic function does not describe the concentration–effect relationship at lower concentrations adequately.

When this is the case, the sigmoid E_{max} equation may be superior to the E_{max} model:

$$E = \frac{E_{max} \times C^n}{EC_{50}^n + C^n}$$

where n is an exponent that changes the shape of the concentration– effect curve. When $n > 1$, the concentration–effect curve is steeper whereas when $n < 1$, the concentration–effect curve is shallower.

With both the E_{max} and sigmoid E_{max} models, the largest changes in drug effect occur at the lower end of the concentration scale. Small changes in low serum concentrations cause large changes in effect. As serum concentrations become larger, further increases in serum concentration result in smaller changes in effect. Using the E_{max} model as an example and setting E_{max} = 100 units and EC_{50} = 20 mg/L, doubling the serum concentration from 5 to 10 mg/L increases the effect from 20 to 33 units (a 67% increase), whereas doubling the serum concentration from 40 to 80 mg/L only increases the effect from 67 to 80 units (a 19% increase). This is an important concept for clinicians to remember when doses are being titrated in patients (Holford and Sheiner, 1981).

1.5.2.1.2. Linear Models

When serum concentrations obtained during a pharmacodynamic experiment are between 20% and 80% of E_{max}, the concentration–effect curve may appear to be linear (figure 1.11). This occurs often because lower drug concentrations may not be detectable with the analytic technique used to assay serum samples, and higher drug concentrations may be avoided to prevent toxic side effects. The equation used is that of a simple line: $E = S \times C + I$, where E is the drug effect, C is the drug concentration, S is the slope of the line, and I is the y intercept. In this situation, the value of S can be used as a measure of drug potency (the larger the value of S, the more potent the drug). The linear model can be derived from the E_{max} model. When EC_{50} is much greater than C, $E = (E_{max}/EC_{50})C = S \times C$, where $S = E_{max}/EC_{50}$.

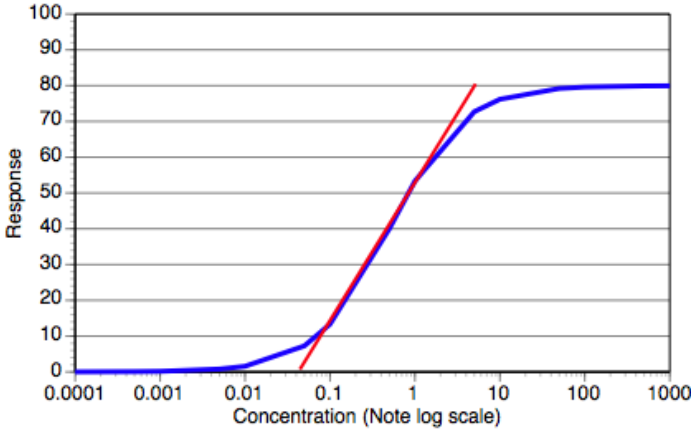

Figure 1.11. The linear model ($E = S \times C + I$) is often used as a pharmacodynamic model (red line).

The linear model allows a nonzero value for effect when the concentration equals zero. This may be a baseline value for the effect that is present without the drug, the result of measurement error when determining effect, or model misspecification. Also, this model does not allow the prediction of a maximum response.

Some investigators have used a log-linear model in pharmacodynamic experiments: $E = S \times (\log C) + I$, where the symbols have the same meaning as in the linear model. The advantages of this model are that the concentration scale is compressed on concentration–effect plots for experiments where wide concentration ranges were used, and the concentration values are transformed so that linear regression can be used to compute model parameters. The disadvantages are that the model cannot predict a maximum effect or an effect when the concentration equals zero. With the increased availability of nonlinear regression programs that can compute the parameters of nonlinear functions such as the E_{max} model easily, use of the log-linear model has been discouraged (Reidenberg, 1999).

1.5.2.2. Pharmacodynamic Interactions

Assessing the nature and the degree of drug interactions is a critical challenge and a debated area of chemotherapy. The importance of drug interactions in chemotherapy is related with the clinical outcome that can be improved or worsened when positive or negative drug interactions, respectively take place (Jankel et al, 1990). Although different terms and classifications can be found in the literature in order to describe drug interactions, three classes can be recognized; 1. positive interaction or synergy. 2. negative interaction or antagonism, which means working against each other (synonyms are depotentiation, desensitization, infrad-ditiveness, negative synergy) and 3. zero-interaction, in which the drugs do neither of the above do not interact and therefore the effect of such a combination is precisely what is expected (synonyms are additivity, indifference, independence, autonomy). The definition of the latter has a central position in drug interaction theories since synergy or antagonism can be defined as departures of the zero interaction and thus a combination is deemed synergistic or antagonistic when its effect is greater or less, respectively than that expected from the zero interaction theory.

For the calculation of the expected effect of zero interaction various theories were developed based upon mechanistic and empirical models (Bernbaum, 1981). The former

models which are based on the mechanism of action of drugs for calculating the expected effects (Carter et al, 1986) require a full understanding of the mode of actions of the drug, are dependent on the current state of knowledge and exclude analysis of the interaction of drugs which modes of action are not known. Even if the mechanisms were fully understood at the molecular level, the effect of a zero interactive combination might be different than expected based on these theories since the action of drugs might be different at the cellular level and at the population level (5) as is the case for susceptibility tests (Bernbaum, 1981). The empirical mechanism-free models rely on concentration-effect curves of individual drugs on which based the effect of zero interaction is calculated without making assumptions about the mechanism of action. Among all theories described in the literature two are distinguished as reference models, the Bliss independence and the Loewe additivity theories.

Bliss independence implies that two drugs do not cooperate physically or chemically or biologically i.e. each drug acts independently from the other (Greco et al, 1995). In this case the effect (S_0) of a zero interactive combination would be equal with the product of the survival fractions S_A and S_B of its constituent doses of drug A and B when acting alone, respectively giving the equation:

$S_0 = S_A \times S_B$ [1].

If the combination of a dose of drug A and a dose of drug B results in a survival fraction of S_{A+B} greater than the S_0 antagonism is deemed while if it is smaller than S_0 synergy is claimed. The equation [1] can be derived from the probability theory. Given that the two drugs do not interact, their actions may be treated as statistically independent events. Thus, if P(A) and P(B) are the probabilities of independent event A and B, the probability of at least one of event A or B occurring $P(A \cup B)$ is

$P(A \cup B) = P(A) + P(B) - P(A) \times P(B)$ [2].

Now, let P(A) and P(B) be the action of two drugs A and B alone (which in case of susceptibility tests corresponds with growth inhibition I_A and I_B caused by each drug, respectively) and the $P(A \cup B)$ the action of a zero interactive combination (I_0) the following equation is derived:

$I_0 = I_A + I_B - I_A \times I_B$ [3].

Since it holds that

$S = 1 - I$ [4]

where S is the percentage of growth and I the percentage of inhibition, the equation [3] is rewritten as $1 - S_0 = (1 - S_A) + (1 - S_B) + (1 - S_A) \times (1 - S_B)$ which after resolution results in equation [1]. Equation [1] is also derived by the following paradigm. Let us consider a drug A that inhibits the growth of a fungal population for 30% (= I_A) and a drug B that inhibits the growth for 50% (= I_B). If the growth of the control is designated as 100%, the drug A alone would reduce the growth to 70% (= S_A). If the drug B now added the growth would be reduced further to 35% (= S_0 and based on equation [4] I_0 = 65%) if the first drug did not affect the action of the second drug. Likewise if drug B is used alone, the growth would be reduced to 50%(= S_B) and the addition of drug A would reduce it to 35% again. Using the values in the brackets equation [1] and [3] come true.

The other candidate for the reference zero interaction theory is the Loewe additivity, which is based on the concept that a drug cannot interact with itself by definition. Therefore, the effect of a sham combination of one drug with itself in any arrangement of concentrations is what would be expected from the concentration effect curve of the drug selected, so such a combination is zero-interactive by construction. This must hold irrespective the shape of the concentration-effect curve of the drug and the type of the effect measured. Loewe additivity is described by the following equation:

$1 = dA/DA + dB/DB$ [5]

where dA and dB are the concentrations of the drugs A and B in a combination which elicits a certain effect and DA and DB the iso-effective concentrations of the drugs A and B when acting alone. When the right part of the equation [5] is less than 1, synergy is claimed while when it is greater than 1, antagonism is concluded. The power of equation [5] is supported by the following paradigm. Lets consider a drug A, 1 mg of which inhibits the growth by 30% and 2 mg by 50%. If the 2 mg powder is split into two portions of 1 mg and labeled as drug A1 and drug A2, the combination of 1 mg of drug A1 plus 1 mg of drug A2 would have the same effect as 2 mg of drug A1 or drug A2 alone which actually is the effect of drug A i.e. 50% of inhibition based on the concentration-effect curve of drug A. Such a combination can only be zero interactive. Thus, for the zero-interactive combination of 1 mg of drug A1 (= dA) and 1 mg of drug A2 (= dB) iso-effective with 2 mg of drug A1 (DA) or drug A2 (DB), the equation [5] is true.

However, the validity of both theories is not undisputed. The equation of the drug actions with statistically defined events and their probabilities in Bliss independence theory

is questioned particularly for complex systems like cells or populations of cells given the high degree of integration and interaction. Therefore, it was suggested that Bliss independence should be applied only in simple systems like single enzymes and simple biochemical pathways. Further-more, using the paradigm of the sham combination of two doses of the same drug, equation [1] holds only when the concentration-effect curves of the individual drugs follow log-linear pattern, otherwise paradoxical results are obtained ((Bernbaum, 1981; Greco et al, 1995). On the other side, Loewe additivity implies that drugs act similarly at the same biochemical site, which is a rare phenomenon, or that the concentration-effect curves of the drugs are the same and therefore it should not be applied when two drugs have different concentration-effect curves. Since neither model can be invalidated based on mechanistic explanations in complex systems such as cells or populations of cells, both models are used to assess drug interactions (Meletiadis et al, 2002).

1.6. Antifungal drugs and their targets

The ideal antifungal agent should have a broad spectrum of activity, low host toxicity, flexible routes of administration, and reasonable cost (Chapman et al., 2008). The target of this ideal agent should be present in a broad spectrum of fungal pathogens, should be essential for fungal cell viability or pathogenicity, but should not be found in human cells to avoid toxicity problems (Carrillo-Muñoz et al., 2006). Finding such a universal target appears to be unrealistic due to the physiological similarities between humans and fungi (Aimanianda and Latgé, 2010). Invasive fungal infections are often difficult to diagnose because their symptoms can be non-specific, and may resemble bacteria or viral infections. Following etiological diagnosis, there are only about two dozen antifungal drugs, which limit therapeutic options. Antifungal drugs can be grouped into the following classes according to their targets.

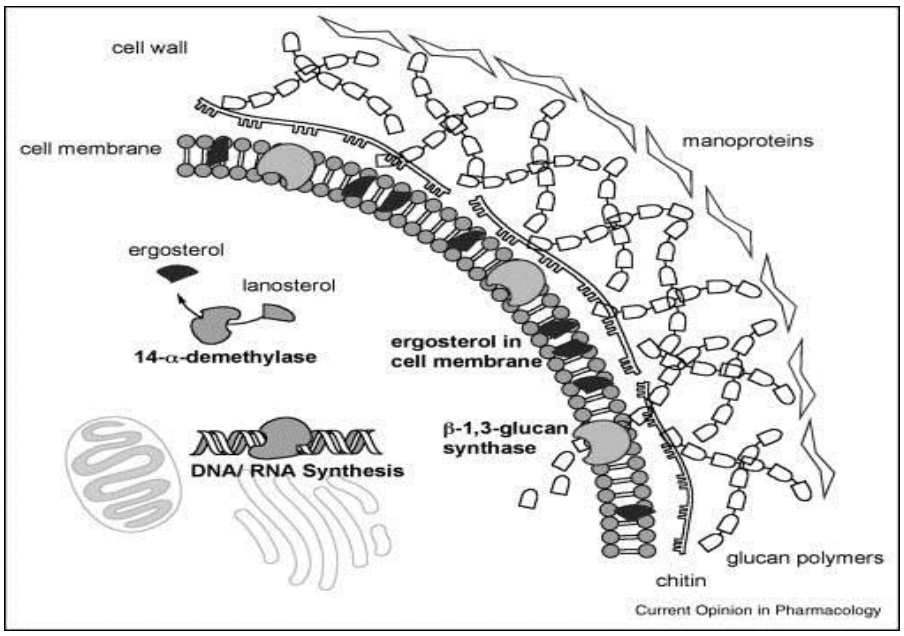

Figure 1.12. Targets of systemic antifungal agents.

Cell membrane targeting antifungals either bind directly to ergosterol (polyenes) or inhibit ergosterol biosynthesis (azoles, allylamines and morpholines). Cell wall targeting antifungals (echinocandins) inhibit wall synthesis (figure 1.12). Other agents, including flucytosine and griseofulvin, target nucleic acid biosynthesis and mitosis respectively (Carrillo-Muñoz et al., 2006; Cannon et al., 2009). No single class of antifungal is effective against all invasive mycoses, and some are highly selective. Each class of drug has a specific mode of action and a distinct role in the treatment of particular fungal pathogens (Chen and Sorrell, 2007).

1.6.1. Polyenes

The polyenes bind irreversibly to ergosterol. Ergosterol is the sterol component of the fungal cell membrane (figure 1.13). It is chemically similar to human cholesterol, and this similarity causes toxicity reactions during treatment. The structure of polyenes allows them to bind to ergosterol, resulting in formation of pores in the membrane and leakage of ions and small molecules that leads to cell death (Bolard, 1986). In addition, polyenes can cause oxidative damage to fungal cell, as shown by *in vitro* studies (Brajtburg et al., 1990).

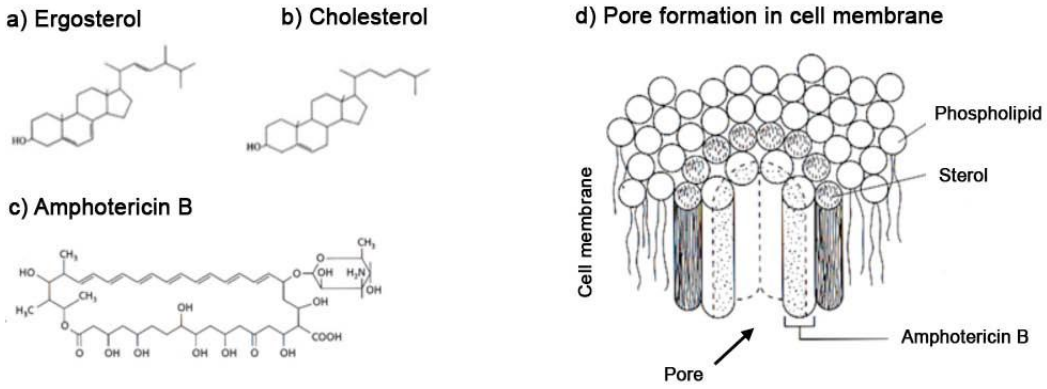

Figure 1.13. Target of polyene drugs. Structures of a) ergosterol, and b) cholesterol, which are found in fungi and mammals, respectively. c) Polyene drugs such as amphotericin B can span a membrane phospholipid bilayer. d) When polyenes are associated with cell membranes, they bind to sterols, and form transmembrane hydrophilic pores.

1.6.1.1. Amphotericin B

Amphotericin B (AMB) is a polyene antimycotic agent (figure 1.13), which has been the mainstay for treating life-threatening, deepseated fungal infections for decades. Its basic mechanism of action is the formation of micellar aggregates with ergosterol, the principal sterol of fungal cell membranes, which leads to an enhanced ion permeability of the membranes and consequently to depolarization and cell death. AMB's broad antifungal spectrum includes almost all human pathogenic fungi (Gallis et al., 1990). Resistance to AMB is rare, apart from a few species that are intrinsically resistant, including *Aspergillus terreus* and *Candida lusitaniae* (Walsh et al., 2003; Rezusta et al., 2008). Unfortunately, AMB clinical use is hindered by its renal toxicity and by the requirement for intravenous administration. Due to its insolubility in water at a physiological pH, AMB was initially used in association with sodium deoxycholate as an emulsifying agent (D-AMB). Three lipid-based formulations of amphotericin B have been developed in order to minimize toxicities associated with AMB. These include amphotericin B lipid complex (ABLC), amphotericin B colloidal dispersion (ABCD), and liposomal amphotericin B (L-AMB). Their development has substantially reduced, but did not eliminate the toxicity completely (Adler-Moore and Proffitt, 2008). A favourable therapeutic response to lipid-based AMB preparations can be expected in 40–60% of patients with IA after approximately 3 weeks of therapy. Comparative clinical studies could not demonstrate superior efficacy of lipid-based AMB formulations over D-AMB (Leenders et al, 1998; Bowden et al, 2002). Likewise, standard

dose regimens of L-AMB were equally effective as high-dose regimens (Ellis et al, 1998; Cornely et al, 2007). However, inhomogeneity of study collectives and varying levels of diagnostic certainty of IA may represent a bias to the study results.

1.6.1.1.1. Pharmacodynamics of AMB

In vitro, AMB formulations display concentration-dependent fungicidal activity with a prolonged post-antifungal effect against *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species (Andes, 2006). In vivo, evidence of the concentration-dependent activity of AMB-D has been confirmed in animal models of acute candidiasis (Andes et al, 2006; Andes et al, 2001) and aspergillosis (Wiederhold et al, 2006) where activity is maximized once peak (C_{max}) plasma drug concentrations surpass the MIC of the infecting pathogen by 4-fold to 10-fold. The concentration-dependent pharmacodynamics of AMB, however, may plateau with repeated dosing because of the limited solubility and high degree of reversible binding of free AMB in tissue, rendering a large portion of the accumulated drug microbiologically inactive (Bekersky et al, 2002). In two postmortem studies, Christiansen et al. (1985) and Collette et al. (1989) found that viable *Candida* and *Aspergillus* isolates could still be recovered from the liver, spleen, and lung of patients treated with AMB, even though tissue concentrations exceeded the MICs of these pathogens by 10–1000-fold. When tissue homogenates were assessed by bioassay and HPLC, bioactive concentrations of AMB represented only 15–41% in the Christiansen study (1985), and 1.5–30% in the Collette study (1989) of total drug concentrations measured by high performance liquid chromatography (HPLC). These findings suggest that only a small and saturable portion of AMB is microbiologically active in tissue, irrespective of the total tissue accumulation or concurrent plasma concentrations of the drug (Lewis and Wiederhold, 2003).

There is a clinical study that has attempted to correlate individual patient-level pharmacokinetics, pathogen MIC, and clinical outcome of L-AMB therapy. Hong et al (2006) examined L-AMB pharmacokinetics and outcome of invasive fungal infections in 39 pediatric oncology patients of age 6 months to 17 years. In a subset of patients (n = 10) for whom detailed pharmacokinetic and MIC data were available, patients with a plasma L-AMB C_{max}/MIC ratio exceeding 40 were more likely to achieve a complete versus partial clinical response (Hong et al, 2006). Interestingly, this threshold PK/PD parameter is in

agreement with experimental PK/PD animal studies that predicted a static effect at a L-AMB plasma C_{max}/MIC 10–20 and maximal activity occurring as the C_{max}/MIC approaches 50 (Andes et al, 2006). For most adults, standard dosages of L-AMB (3–5 mg/kg) should surpass a C_{max}/MIC of 40 unless the pathogen has an AMB MIC of 2 mg/mL or greater (Andes et al, 2006).

1.6.1.1.2. Tolerability and Adverse effects of AMB

D-AMB is well known for frequently causing acute and potentially lethal infusion-related reactions involving symptoms like fever, chills, hypotension, dyspnoea, tachycardia, headache and nausea, which are thought to be triggered by the liberation of inflammatory mediators via stimulation of toll-like receptors and the induction of a CD14-dependent mechanism (Sau et al, 2003; Goodwin et al, 1995). Although the concomitant administration of anti-inflammatory drugs may mitigate these symptoms, and the evolution of a certain tolerance may be observed later in the course of therapy (Goodwin et al, 1995), great effort was laid on the development of better tolerated lipid-associated formulations of AMB. However, among these, only L-AMB showed a significant benefit in the prevention of infusion-related toxicity compared to D-AMB (Bowden et al, 2002; Walsh et al, 1998; Walsh et al, 1999). In addition to infusion-related adverse effects, AMB may be associated with considerable cumulative toxicity like cardiotoxicity (Googe and Walterspiel, 1988; Danaher et al, 2004), neurotoxicity (Walker and Rosenblum, 1992) and, most notably, nephrotoxicity (60–80% of patients), the latter manifesting in tubular injury and renal vasoconstriction (Fanos and Cataldi, 2000). Although AMB's nephrotoxic effects are to a certain extent preventable (e.g. by sodium supplementation) and reversible (Anderson, 1995), they represent the main dose-limiting determinants. Fortunately, all approved lipid-based formulations were shown to significantly reduce the likelihood of severe azotaemia compared to conventional D-AMB, even in patients treated concomitantly with other nephrotoxic drugs (Leenders et al, 1998, owden et al, 2002; Walsh et al, 1999). Thus, in many hospitals, conventional D-AMB, despite its lower cost, is largely abandoned as a therapeutic agent against IA (Kleinberg, 2006). Despite its unfavourable safety profile, AMB still represents the best proven and most important therapeutic option in salvage situations and in the management of breakthrough infections.

1.6.2. Azole antifungal agents

Azoles are the most widely used as well as the best-studied class of antifungal drugs (Sheehan et al., 1999). In addition, azoles are abundantly used in the environment as agricultural pesticides for plant fungal diseases (Verweij et al., 2009). Azoles act by inhibiting the fungal cytochrome P450 enzyme (also known as 14α-sterol demethylase or lanosterol demethylase). This depletes cellular ergosterol and causes accumulation of toxic sterol intermediates (Sheehan et al., 1999) (figure 1.14). There are two groups of azoles in clinical use: imidazoles and triazoles. Azoles are generally fungistatic against yeast, although some triazoles have fungicidal activity against moulds (Francois, 2006).

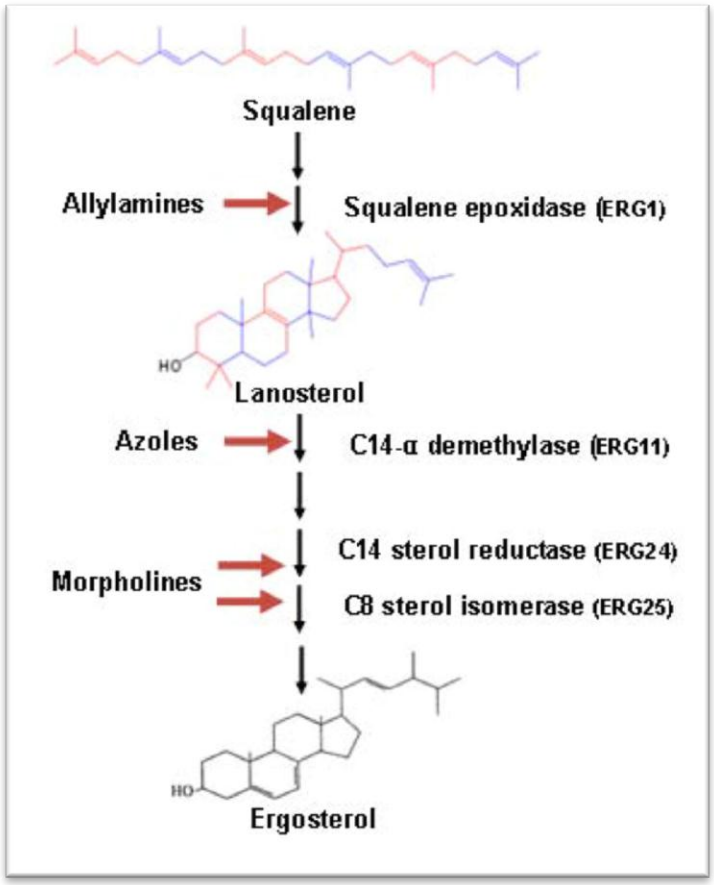

Figure 1.14. Antifungal drugs that target ergosterol biosynthesis. Azoles inhibits ergosterol biosynthesis at different steps. Azoles bind C14 α -demethylase (ERG11).

1.6.2.1. Imidazoles

The imidazoles have a five membered ring that includes two nitrogen atoms. The imidazoles in clinical use include ketoconazole, miconazole, clotrimazole, and econazole. Except for ketoconazole, imidazoles can only be applied topically due to their human toxicity, so their use is limited to treating superficial mycoses (Zhang et al., 2007).

1.6.2.2. Triazoles

The triazoles have a five membered ring with three nitrogen atoms (figure 1.15). There are two generations of triazoles: the first generation (fluconazole and itraconazole), and the extended-spectrum second generation (voriconazole and posaconazole), which have a more extensive range. Triazoles have greater affinity for fungal compared with mammalian cytochrome P450 enzymes, so their safety profile is significantly improved for systemic use.

Fluconazole is active against yeast but not against *Aspergillus* and other moulds (Koltin, 1997), so it is mainly used for treating oral candidiasis. However, development of fluconazole-resistant *Candida* strains has become a major issue in treatment of many patients (Tumbarello et al., 2009). Voriconazole and posaconazole have broad-spectrum activity against yeasts and moulds, including *Aspergillus*. Voriconazole is currently the drug of choice for the management of invasive aspergillosis. Posaconazole is the only azole drug with activity against zygomycete fungi. In general, the triazoles are relatively safe, even when used for prolonged periods, although they can cause liver toxicity (Spanakis et al., 2006)**.**

1.6.2.2.1. Voriconazole

1.6.2.2.1.1. Efficacy of Voriconazole in the Therapy of IA

Voriconazole demonstrate good activity against *Aspergillus* strains, even when resistant to AMB and itraconazole. As is the case for all triazole antifungal agents, voriconazole catalyses a key step in the membrane synthesis, namely the conversion of lanosterol to ergosterol (Sutton et al, 1999) (figure 1.14).

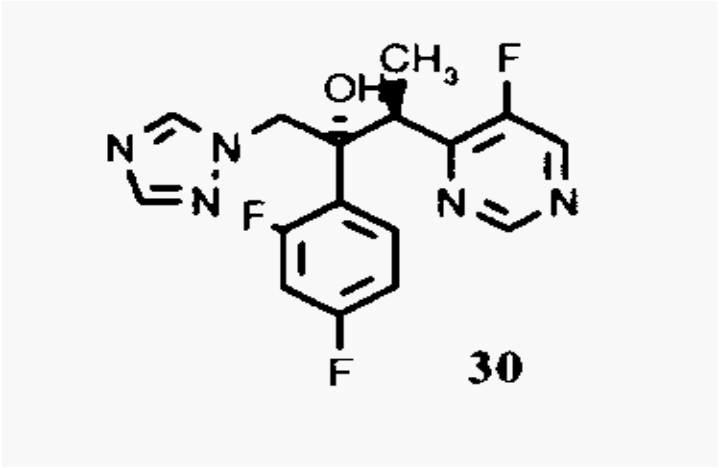

Figure 1.15. The chemical structure of Voriconazole

Voriconazole can be given both intravenously and orally, allowing for dose flexibility and ambulatory treatment, if clinically reasonable. Factors that render the plasma concentrations of voriconazole highly unpredictable include genetic polymorphism of the hepatic cytochrome P-450 (CYP) isoenzyme CYP2C19, liver dysfunction, infancy and, in general, the non-linear pharmacokinetics of voriconazole in adults (Li-Wan et al, 2010; Brüggemann et al, 2009; Walsh et al, 2004). Several authors, therefore, suggest therapeutic monitoring of drugs metabolized by CYP2C19, as well as voriconazole itself, as long as voriconazole therapy is continued (Brüggemann et al, 2009; Pascual et al, 2008). Corresponding to its excellent in vitro activity against *Aspergillus* spp., voriconazole has shown encouraging clinical efficacy in animal models of IA (Herbrecht, 2004).

Patients with a broad range of immunocompromising disorders complicated by proven or probable IA were randomly allocated to receive either voriconazole (4 mg/kg twice daily) or D-AMB (1–1.5 mg/kg once daily). At the earliest, after 7 days the intravenous voriconazole therapy could be switched to oral administration of 200 mg twice daily, if clinically reasonable. The superiority of voriconazole therapy was statistically significant and is confirmed in a retrospective stratification study (Ali et al, 2006; Vehreschild et al, 2010; Herbrecht et al, 2002). Variables assessed include the level of certainty of IA diagnosis (as assessed by independent investigators), the underlying condition, the site of infection and the leucocyte counts. Likewise, the overall survival rate was higher in the voriconazole group (71%) as compared to the D-AMB group (58%; p=0.02), even though the voriconazole group included significantly more cases of proven IA. The authors discussed a possible bias from the longer treatment duration in the voriconazole group. It was emphasized, however, that for a successful outcome in IA, the early treatment is crucial (Ali et al, 2006; Vehreschild et al, 2010; Herbrecht et al, 2002). Fewer patients in the voriconazole group had to be given another licensed antifungal therapy compared with patients of the D-AMB group (36 vs. 80%) (Patterson et al, 2005). The rates of complete or partial response in acute IA range from 41 to 53% after approximately 80–100 days of therapy. Over the course of voriconazole therapy, the time of intravenous administration seldom exceeded 14 days. Of note, the overall length of therapy was not related to the emergence and severity of adverse events. Discontinuation due to drug intolerability occurred, if only rarely, mainly in the first days of therapy. Distinct studies, however, indicated a direct correlation between low plasma trough

concentrations and therapeutic failures (Denning et al, 2002; Smith et al, 2006). Therefore, therapeutic drug monitoring, if feasible, seems especially advisable in children and multimorbid patients. Due to its good ability to penetrate the cerebrospinal fluid, voriconazole is also the first choice in cerebral aspergillosis (Böhme et al, 2003).

1.6.2.2.1.2. Pharmacodynamics of voriconazole

Studies with voriconazole have shown that increasing drug concentrations do not enhance antifungal effect (a wide concentration range (starting below the MIC [sub- MIC] to those more than 200-fold in excess of the MIC), growth of *Candida* organisms are similarly inhibited (Andes and Van Ogtrop, 1999). Furthermore, in vitro studies demonstrated organism regrowth soon after drug removal (Ernst et al, 2000; Turnidge et al, 1994). In vivo studies, however, demonstrated prolonged growth suppression after levels in serum decreased to below the MIC (Andes and Van Ogtrop, 1999; Andes et al, 2003). These prolonged in vivo post antifungal effects (PAFEs) have been theorized to be caused by the profound sub-MIC activity of these drugs (ie, effect of the voriconazole after concentrations fall below the MIC in vivo). The time kill combination of concentration-independent killing and prolonged PAFEs suggest that the 24 AUC/MIC parameter is most closely tied to treatment effect. Dose fractionation studies in several in vivo models with voriconazole have corroborated these results (Andes et al, 2003). The results clearly demonstrated that outcome depended on the total amount of drug or AUC rather than the dosing interval. Subsequent studies with voriconazole similarly demonstrated that outcome was independent of fractionation of the total drug exposure supporting the 24-hour AUC/MIC as the pharmacodynamic parameter driving treatment efficacy (Andes et al, 2003). These later observations demonstrate that the pharmacodynamic parameter associated with efficacy was similar within the voriconazole drug class.

The usefulness of knowing which parameter predicts efficacy is being able to then determine the magnitude of that parameter needed for successful outcome. The most efficient experimental way to define the magnitude of the predictive parameter is to examine treatment efficacy against *Aspergillus* species with widely varying MICs. Results from these studies showed that the AUC/MIC exposure associated with treatment efficacy was similar across the group of strains. Similar studies have now been undertaken with

four triazole compounds that include more than nearly 40 drug/organism combinations for which MICs and dose levels varied more than 1000-fold each (Andes et al, 2003).

Most recently attempts have been made to similarly correlate the pharmacokinetics of the recently approved triazole, voriconazole, with MIC, and outcome (Pfaller et al, 2006). If one considers the kinetics of voriconazole in humans, an intravenous dose of 4 mg/kg every 12 hours would produce free drug AUCs of approximately 20 mg.h/ml. Given a pharmacodynamic target of a free drug AUC/MIC ratio of 20–25, one could predict that these voriconazole dosing regimens could successfully be used for treatment of infections caused by *Candida* spp. for which MICs are as high as 1 mg/L. Indeed, maximal efficacy was observed with *C. albicans* isolates for which MICs were less than 1 mg/L. The highest failure rates (45%) were observed with *C. glabrata* isolates for which many MICs were greater than 1 mg/L. These data were used in the development of susceptibility breakpoints for voriconazole (Pfaller et al, 2006).

Unfortunately there are no complete clinical databases (kinetics, MIC, and outcome) to examine these relationships for voriconazole or other antifungals in treatment of filamentous fungal infections. There is, however, an accumulating body of evidence from which one can attempt to draw pharmacodynamics information. There have been more than 40 reported patients who have developed breakthrough infections while receiving voriconazole (Alexander et al, 2005; Imhof et al, 2004; Marty et al, 2004; Siwek et al, 2004; Smith et al, 2006). A common feature of nearly all of these cases was infection with an organism for which the voriconazole MIC was greater than 1 mg/L. Unfortunately voriconazole serum concentrations were not available for these patients. A recent case series did, however, identify a relationship between voriconazole serum concentration and patient outcome (Smith et al, 2006). Patients who have concentrations less than 2 mg/L were more likely to die from invasive fungal infection (mostly aspergillosis) than those who had serum concentrations exceeding this value. Considering free drug concentrations and the MICs of organisms involved in these case series, one can estimate that treatment failure was associated with 24-hour free drug AUC/MIC values less than 20 to 50.

1.6.2.2.1.2. Adverse Effects of Voriconazole Therapy

Adverse side effects of voriconazole are usually mild and transient. The most noted are visual disturbances in the form of blurred vision, photophobia or altered colour

perception. In clinical studies, these side effects occurred during the first week of therapy, yet rarely led to withdrawal of voriconazole treatment (Walsh et al, 2004, Herbrecht, 2004). An elevation of liver enzymes is reversible with few exceptions reported in patients with pre-existing hepatic dysfunction and poor general health (Herbrecht, 2004). However, it is difficult to unequivocally attribute elevated liver enzymes to voriconazole therapy, because most of the critically ill patients receive a variety of comedication and may suffer from dermal manifestations of their underlying disease. Less frequent adverse findings include headaches, hypoglycaemia, hallucinations, 'drunken' feeling, rash, facial erythema, cheilitis and increased photosensitivity (Herbrecht, 2004; Boyd et al, 2004). The latter was made responsible for cutaneous squamous cell carcinomas and melanomas emerging in patients after ultra long-term voriconazole ingestion (1-12 months) (McCarthy et al, 2007; Cowen et al, 2010; Miller et al, 2010; Morice et al, 2010). The occurrence of adverse side effects of voriconazole has been shown to positively correlate with increasing plasma concentrations (Pascual et al, 2008; Boyd et al, 2004; Howard et al, 2008).

1.6.3. Echinocandins

The echinocandins are the newest class of antifungals. These compounds target the cell wall by inhibiting β -1,3-glucan synthase (figure 1.12). Caspofungin was the first echinocandin approved and used clinically in 2001, followed shortly by micafungin and anidulafungin (Morrison, 2006). Echinocandins show good activity against *Candida* and *Aspergillus*, but they are not effective against *Cryptococcus neoformans* or non-*Aspergillus* moulds. Echinocandins are fungicidal against most ascomycete yeasts, but are fungistatic against most moulds with strong efficacy *in vivo* (Bowman, 2002)**.** Because echinocandin action is specific to fungal cell walls, human toxicity associated with echinocandins is limited (Gauwerky et al., 2009). However, all echinocandins require injection, as there are no oral preparations available (Kauffman and Carver, 2008).

1.6.3.1. Caspofungin

1.6.3.1.1. Efficacy of Caspofungin in IA

Like all echinocandins, caspofungin is a high-molecular-weight lipoprotein and can thus be administered by intravenous infusion only (figure 1.16). Due to its protein structure, its ability to penetrate into the cerebrospinal fluid is poor. In all prospective

studies, caspofungin was given at a loading dose of 70 mg on day 1, followed by once daily maintenance doses of 50 mg for a median of 2–4 weeks. The earliest publications reported the use of caspofungin as salvage therapy. All *Aspergillus* species isolated were highly susceptible to caspofungin. At the individual end of therapy, a favourable outcome was observed in 5 patients including one with cerebral involvement. In addition, caspofungin was well tolerated in paediatric population (Zaoutis et al, 2009). It is worth mentioning that, in salvage studies, radiographic refractoriness after only 1 week of first-line therapy is frequently accepted as an inclusion criterion (Walsh et al, 2007; Walsh et Al, 2002; Maertens et al, 2004; Zaoutis et al, 2009). However, this practice may skew the interpretation of efficacy data, as, in computer tomography examinations, pulmonary lesions may initially increase in volume despite the effectiveness of the first-line therapy (Caillot et al, 2010). In 2009, caspofungin was evaluated in an open, multicentric phase II study as a first-line therapy in IA.

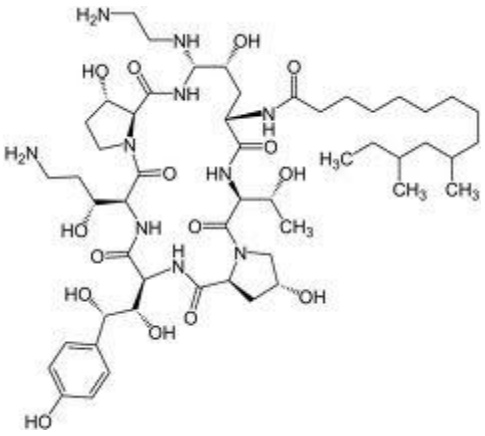

Figure 1.16. The chemical structure of caspofungin.

The acute leukaemia and neutropenia at baseline were independent prognostic factors tendentially associated with lower response rates (Viscoli et al, 2009). This special patient population is well known for being at the highest risk to die from IA (Nivoix et al, 2008; Garcia-Vidal et al, 2008).

1.6.3.1.2. Caspofungin pharmacodynamics

Caspofungin is slowly metabolized by hydrolysis and N-acetylation in the liver (Stone et al, 2002), it exhibits concentration-dependent killing with a prolonged post-

antifungal effect against Candida species (Andes, 2006). In the experimental animal models of candidiasis (Andes et al, 2003; Louie et al, 2005; Gumbo et al, 2006) and aspergillosis (Wiederhold et al, 2004), caspofungin activity is optimized when plasma C_{max}/MIC ratio approaches 10 or the tissue 24-hour area under the curve to MIC ratio (AUC/MIC) exceeds 250 (Louie et al, 2005). Like AMB formulations, supra-therapeutic concentrations of the caspofungin may persist in tissue even when plasma concentrations fall below the MIC (Louie et al, 2005). This characteristic, along with the safety and concentration-dependent pharmacodynamic properties of this class, has stimulated interest in infrequently administered (i.e. 1–3 weekly), high-dose caspofungin regimens.

Interestingly, a paradoxical effect has been observed for caspofungin against some *Candida* and *Aspergiilus* species, whereby higher drug concentrations fail to result in a greater degree of killing compared with concentrations at or near the MIC (Clemons et al, 2006; Stevens et al, 2004; Stevens et al, 2005). Investigations of isolates that are able to paradoxically sustain growth in the presence of high caspofungin concentrations have revealed rapid and transient induction of genes involved in cell wall homeostasis, as well as an increase in cell wall chitin concentrations. The clinical significance of this paradoxical phenotype is still unknown and is an area of intensive research.

Caspofungin is a most promising compound to be utilized in human IA. In clinical studies employing strict diagnostic criteria for IA, approximately 40% of patients responded favourably. Based on these results, at the 3rd European Conference on Infections in Leukaemia (2009), caspofungin for primary therapy of IA was upgraded from evidence CIII to CII (Herbrecht et al, 2009). However, the rate of complete remissions in respective studies was low. Consequently, at present caspofungin is licensed for the secondline therapy of IA and for the empirical therapy of presumed fungal infections in patients with febrile neutropenia. The greatest advantage of caspofungin is its excellent tolerability which has been confirmed in clinical trials (Viscoli et al, 2009; Herbrecht et al, 2010; Maertens et al, 2004; Zaoutis et al, 2009).

1.6.3.1.3. Adverse effects of Caspofungin

Adverse events attributed to caspofungin in clinical trials were usually mild, unspecific symptoms like headache, fever or gastro-intestinal symptoms. Severe adverse events judged to be drug related were rarely observed in children and adults. The most

common laboratory findings include mild-to-moderate elevation of the levels of hepatic transaminases and creatinine and haematological abnormalities (Viscoli et al, 2009; Herbrecht et al, 2010; Maertens et al, 2004; Zaoutis et al, 2009). In contrast to azoles, caspofungin shows little effect on the cytochrome P-450 enzyme system and does not interact with p-glycoprotein (Wagner et al, 2006). Thus, the potential for clinically significant interactions with co-administered drugs is low.

1.6.4. Flucytosine

Flucytosine (5-fluorocytosine; 5-FC) is a fluorinated analogue of the nucleotide base cytosine. It was synthesized in 1957 as a potential antitumor agent, but it was also used in 1968 for treatment of human candidiasis and cryptococcosis (Vermes et al., 2000). 5-FC is imported into cells by the action of the enzyme cytosine permease. In the cell, 5-FC is converted into the metabolically active nucleoside analogue 5-fluorouracil, which inhibits RNA and DNA synthesis. 5-FC should not be administered as a single agent because of rapid development of resistance (Barchiesi et al., 2000). Also, 5-FC should be used with caution in patients with renal insufficiency. 5-FC has very limited spectrum of activity (*Candida* species*, Cryptococcus neoformans*, some moulds). Its role is clinically limited the administration in combination with AMB for treating cryptococcal meningitis (Patel, 1998).

1.6.5. Allylamines

The allylamines inhibit an early stage of ergosterol biosynthesis by binding the enzyme squalene epoxidase (figure 1.7). Terbinafine is the only allylamine in clinical use. As it accumulates in hair, nail and skin, topical and oral preparations of terbinafine are widely used to treat nail and skin infections such as tinea pedis. Terbinafine displays a primary fungicidal action against dermatophytes and filamentous fungi (Petranyi et al., 1987). There are also new reports of its value in certain invasive mould infections in combination with either a triazole or AMB formulation (Yu et al., 2008). Terbinafine is generally well tolerated, but may cause gastrointestinal upset and transient elevation of liver enzymes.

1.6.6. Morpholines

There is only one morpholine used clinically, amorolfine**.** It blocks two enzymes in ergosterol biosynthesis (figure 1.7). These are the sterol C8-C7 isomerase and the C-14 sterol reductase, which result in ergosterol depletion. Amorolfine is active against dermatophytes and yeasts, but it can only be applied topically (Gauwerky et al., 2009).

1.6.7. Griseofulvin

Griseofulvin is an oral antifungal agent that was first used to treat superficial fungal infections of hair, nail and skin (Bedford et al., 1960). Griseofulvin has been the treatment of choice for the skin infection *tinea capitis* (ringworm) for more than 40 years (Gupta et al., 2008). Griseofulvin binds to microtubules where it acts mainly by inhibiting mitosis. Notably, the griseofulvin concentration required to inhibit the growth of fungal cells is much lower than that required to inhibit the mammalian cells due to its higher affinity for fungal tubulin (Panda et al., 2005). Griseofulvin has recently been shown to inhibit proliferation of various types of cancer cells and to inhibit tumor growth (Rathinasamy et al., 2010).

1.7. Problems associated with antifungal therapy

Despite the availability of effective antifungal agents including AMB, echinocandins and second-generation triazoles, therapy failure is a clinical problem with all invasive fungal infections. Therapy failure was reported in 40–70% of patients with invasive aspergillosis (Nucci and Perfect, 2008). Overall, numerous problems are associated with current antifungal therapies. The main problems are host toxicity, narrow activity spectra, and evolved resistance(Chapman et al., 2008). Collectively these lead to high economic costs for treatment of fungal infections, as well as increased mortality rates. The problems associated with current antifungal drugs demand the development of new antifungal therapeutics.

1.7.1. Emerging resistance

Intrinsic and acquired antifungal resistance is a growing clinical problem with many fungal pathogens. Antifungal drug resistance can be caused by: 1) mutation or

overexpression of the drug target, 2) biofilm formation, 3) drug efflux by upregulation of the efflux pumps, 4) stress-related tolerance that enhances short-term survival, or 5) modification of chromosomal ploidy (Monk and Goffeau, 2008; Marie and White, 2009).

Resistance to amphotericin B is still rare but insite of that the lipid complex and liposomal formulations have been developed to improve tolerability for the patient with fungal infection. However, the resistance to amphotericin B may not be related to the minimum inhibitory concentration (MIC), but to failure of the antifungal agent to penetrate into infected tissue (Espinel-Ingroff and Pfaller, 2007; Kanafani and Perfect, 2008).

The extensive prophylactic use of fungistatic azole derivatives, especially fluconazole, is associated with the emergence of resistant fungal pathogens (Canuto and Rodero, 2002). Furthermore the use of azoles in agriculture has led to development of cross-resistance to related azoles that are used as drugs. This was recently found in *A. fumigatus* clinical isolates (Verweij et al. 2009). Resistance to azoles is an important factor in treatment failure. Some filamentous fungi can cause infections that are unresponsive to current therapies and so can be lethal. Antifungal drug resistance also has striking economic consequences. A relationship has been reported between C. albicans resistance to azole (increased expression of MDR1 gene) and its increased experimental pathogenicity (e.g., more rapid and extensive hyphae formation, an increased adherence to plastic and the propensity for biofilm formation), which could have an adverse effect during prolonged, dosage-increased treatment with both azole and echinocandin antifungal agents (Angiolella et al, 2008). Although resistance to the triazoles is not common among *Aspergillus* spp. (Da Silva Ferreira et al, 2005; Espinel-Ingroff and Pfaller, 2007). Two mechanisms of resistance to azoles have been described for Aspergillus spp.: either point mutations of Cyp51A or reduced concentration of intracellular drug. In addition, the overexpression of efflux pumps as an azole resistance mechanism has been observed mostly in *A. fumigatus* mutants (Espinel-Ingroff, 1998; Kanafani and Perfect, 2008).

Development of resistance to caspofungin also is a rare event. However, the species with higher MICs may predispose these strains to resistance development. On other hand, the paradoxical phenomenon or the "eagle effect" that some mould isolates are able to grow at echinocandin high concentrations above the MIC and this is the

modest drug resistance or drug tolerance due to an adaptive cellular physiology (Perlin, 2007; Stevens et al, 2005).

1.7.2. Low efficacy

A narrow spectrum of activity against some fungal pathogens is related to some classes of antifungals. 5-FC is active against yeasts such as *Candida* and *Cryptococcus* but has limited activity against moulds. Terbinafine is the drug of choice for treatment of dermatophyte infections but it has limited activity against invasive fungal infections. Echinocandins are effective against *Candida* and *Aspergillus* species, but not against *C. neoformans* or non-*Aspergillus* moulds (Denning and Hope, 2010).

1.8. The fungal cell wall components

The fungal cell wall is a three-dimensional mesh work composed of several types of polysaccharide and glycoprotein (Aimanianda and Latgé, 2010) (figure 1.17). It corresponds to about one quarter of fungal cell biomass (Gastebois et al., 2009); about one third of the fungal genome (~ 4000 genes) is involved in cell wall biosynthesis and maintenance (de-Groot et al., 2009). Fungal genomes typically have suites of genes with partially redundant and overlapping functions that encode synthetic enzymes for wall formation and others for its maintenance (Latge, 2007).

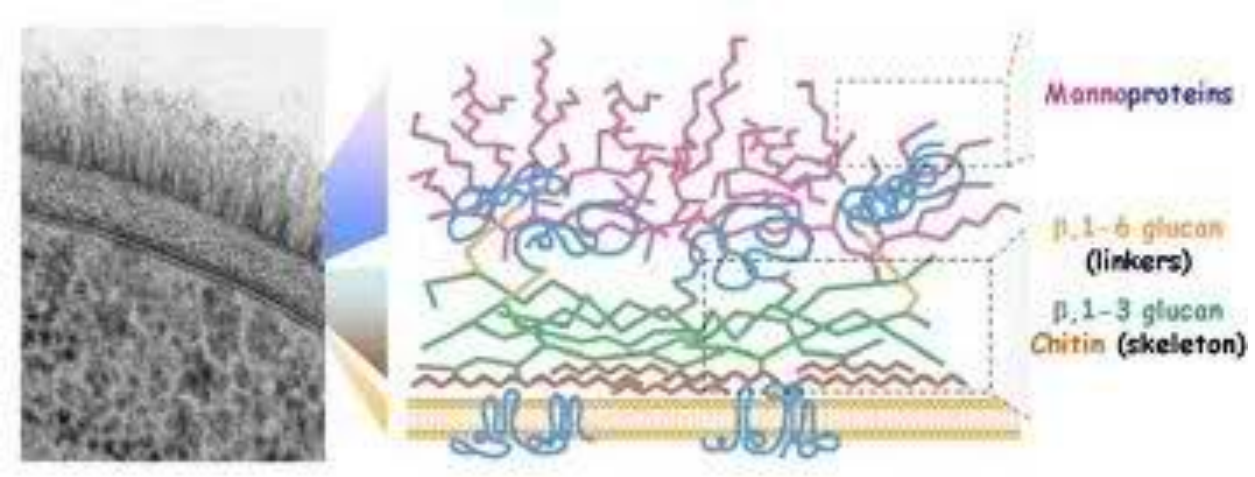

Figure 1.17. The composition and architecture of fungal cell wall in filamentous fungi

The fungal wall functions include: 1) environment sensing and protection from certain environmental stressors, 2) adhesion to and penetration of host tissues, and 3)

mediating secretion of pathogenicity factors and hydrolytic enzymes. Disruption of the cell wall renders the fungus susceptible to lysis or death (Aimanianda and Latge, 2010).

Despite decades of effort to characterize the composition and architecture of fungal cell walls (Bowman and Free, 2006), many details remain poorly understood (figure 1.17). In part, this is because analysis of biologically distinctive carbohydrates is hampered by their similar chemistry. In filamentous fungi, the polysaccharides form about 90 % of the cell wall, which consists mainly of glucans, chitin and galactomannan (Gastebois et al., 2009). Glucans are the major fungal cell wall polysaccharides, constituting about 50-60 % of the wall dry weight. Amongst these, β-1,3-glucan predominates, with α-1,6-glucans and β-1,3-glucans also present in lower amounts. Chitin, a α-1,4 polymer of N-acetylglucosamine, accounts for 10-20 % of fungal cell wall mass. In *Aspergillus* cell walls, galactomannan accounts for 20-25 % of the polysaccharides (Gastebois et al., 2009).

Galactomannans are branched polymers of carbohydrate that are found in many fungi including *Aspergillus* (Latge et al., 2005). As well as being a part of the fungal cell wall, Galactomannans is the major antigen found in the bodies of patients suffering from aspergillosis (Leitao, 2003). The immunodominant epitope in the galactomannans molecule is galactofuranose. This has been the basis of the development of a monoclonal antibody that detects galactomannans presence in serum, bronchoalveolar lavage or CSF of patients with invasive aspergillosis (Mennink-Kersten et al., 2004).

1.9. Assessment of antifungal drugs efficiency

Antifungal susceptibility testing (AFST) is an *in vitro* method used to predict whether a specific antifungal drug can be used to treat succesfully an infection caused by the fungal isolate of a fungus to being examined. AFST was less well developed and utilized than antibacterial testing (Lass-Flörl, 2006). The requirement for accurate and predictive susceptibility testing of fungi did not become a major issue until the start of the AIDS epidemic, which caused dramatic increase in the incidence of fungal infections (Johnson, 2008). Recently, AFST has been standardized for both yeasts and filamentous fungi. Two international standard methodologies are available: the Clinical and Laboratory Standards Institute (CLSI) method (Espinel-Ingroff et al., 2005), and European Committee for Antimicrobial Susceptibility Testing (EUCAST) method (Lass-Flörl, 2006).

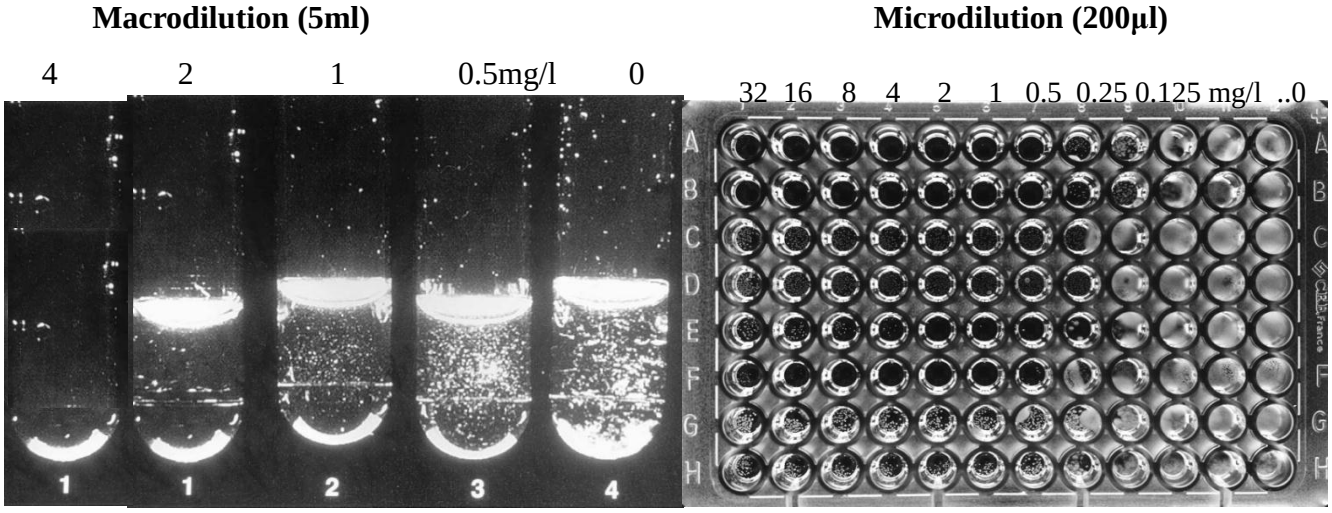

Figure 1.18. In vitro susceptibility testing methodology

Both of these methods are broth-microdilution based assays (figure 1.18) and both have good inter- and intra-laboratory reproducibility (Lass- Flörl et al., 2010). Broth dilution methods use the minimum inhibitory concentrations (MICs) of antifungal drugs as an endpoint to define an appropriate dose. MIC is defined as the lowest concentration of the drug that inhibits the growth of fungi within a defined period of time. MIC is expressed as mg/L and must be observed visually (Subcommittee on Antifungal Susceptibility Testing (AFST) of EUCAST, 2008) (figure 1.12).

Assessment of *in vitro* activity of echinocandins against *Aspergillus spp*. is complicated because of the phenomena of trailing endpoints and the fact that MIC values are usually higher than the allowed treatment doses, which makes MICs for *Aspergillus* poorly reproducible. In order to deal with these difficulties, the minimum effective concentration (MEC) is used as an end point for echinocandins, since it generates more consistent susceptibility results than MIC. MEC is defined as the lowest drug concentration at which short, stubby, highly branched hyphae are observed by microscopic examination (Espinel-Ingroff et al., 2007).

1.9.1. Clinical and Laboratory Standards Institute (CLSI), method for Moulds (M38-A2)

A new mould method, CLSI M38-A2, was released in 2002. This method is nearly identical to M27-A3 with the exception of the inoculum size. The inoculum size is determined spectrophotometrically but to a higher desired final concentration of 0.4–5 × 10^4 CFU/ml. The guideline provides target percent transmission (%T) readings based on

conidial size that are listed by species. Isolates from the genera of *Aspergillus* spp., *Paecilomyces* spp., and *Sporothrix* spp. are measured at 80–82%T while species with larger conidia such as *Fusarium* spp., *Rhizopus* spp., and *Scedosporium* spp. are standardized to 68–70%T (Espinel Ingroff et al, 1997). Efforts are under way to determine the correct %T for most of the clinically significant fungi, but the list is not yet complete. When necessary, mould testing may be conducted by the macrobroth method (figure 1.18) as early studies have shown that the two methods are equivalent. Other fungi that may benefit from testing by the macrobroth method are those fungi that grow very slowly. It is difficult to hold microtiter tests longer than 72h due to dehydration. Many of the less frequently encountered fungi may require as long as 120–144h before growth is detected in the drug-free growth control well. For this reason, isolates that are known to be slow growers should be tested via the macrobroth method (Pasarell and McGinnis, 1992).

Endpoint determination is also much more difficult with moulds than with the yeast fungi. While a reduction in turbidity is typically easy to visualize with yeast fungi, it is not so easily visualized when moulds are tested. Due to the unique growth patterns of the mould fungi, one looks for a decrease in volume of growth rather than a reduction in turbidity as for the yeasts. *Aspergillus* spp. growth is seen as a cottony clump in the broth. To determine an endpoint, the reader must assess the amount of growth for each concentration and call the endpoint at that concentration that has at least 50% smaller volume of growth for antifungals not read at 100% inhibition. Many individuals are not comfortable with this subjective endpoint determination and prefer to refer mould testing to reference centers. Reading the MIC endpoint for moulds differs from the criteria established for the yeast fungi. Amphotericin B, itraconazole, posaconazole, and voriconazole endpoints are all determined at the lowest concentration that prevents discernible growth or in other words, the first clear well. The echinocandins do not provide a MIC but rather a MEC, or minimum effective concentration. The echinocandins attack the growing tips of the hyphae resulting in aberrant, stubby growth of the hyphae. This aberrant growth is easily visualized as the hyphae cluster within the well in clumps. The MEC is the lowest concentration where the growth within the well is visually clumped. Microscopic examination will display obviously distorted hyphae. Work has not been completed that permits categorizing moulds as susceptible or not. General guidelines have been established to assist with analyzing mould data (CLSI, 2008). Based on large

amounts of data (Lass-Florl et al, 1998), isolates are considered susceptible to amphotericin B, itraconazole, posaconazole, voriconazole, and caspofungin when the MIC/MEC is 1.0 mg /ml, intermediate with MIC/MEC is 2.0 mg /ml, and resistant when the MIC/MEC is 4.0 m g/ml. It is likely that the other echinocandins would fit into these ranges as well.

1.10. Towards more effective antifungal therapy (drug combination)

The high rate of mortality from mould infections and the relatively limited number of effective antifungal drugs have produced pressing needs for new therapeutic options. This has led to the use of drug combination, especially to treat intractable infections (Kontoyiannis and Lewis, 2004). There are several possible benefits for using two or more antifungal drugs, instead of monotherapy. Combination therapy may achieve fungicidal activity that is not possible with only one agent. Drug combinations may be effective at lower drug dosages, thus reducing toxic side-effects while also increasing efficacy. Combination therapies may delay or possibly prevent the emergence of drug-resistant mutants. In addition, combination therapy may provide broader spectrum coverage for seriously ill patients (Wirk and Wingard, 2008).

When two drugs are used together, drug interaction can be classified as synergistic, additive/indifferent or antagonistic on the basis of the fractional inhibitory concentration (FIC) index. The combined FIC is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone (Te Dorsthorst et al., 2002). In case of synergism, the combined effect of two drugs is greater than the sum of the individual effects. Additive interaction means the effect of two drugs is equal to the sum of the effect of them when taken separately. In case of antagonism the combined effect is less than the sum of the effects produced by each drug separately (Kontoyiannis and Lewis, 2003).

Numerous in vitro investigations of various combinations of antifungal agents and trials in animal models of fungal infection have been performed (Johnson and Perfect, 2010). However, most of these antifungal combinations have not been evaluated in large clinical trials, in part because of the time and expense involved (Marr, 2004, Ashley and Johnson, 2011).

An additional problem with combination therapy is lack of generality of effect: combinations that prove successful with certain infections may not be broadly effective in improve the therapeutic outcome in others. A example to that constitutes the established, therapy for cryptococcal meningitis where 5-FC used in combination with polyenes or azoles (Van et al., 1997).

1.11. In Vitro Dynamic Mycotic Infection Model

In vitro kinetic system can be used to identify new agents with noval pharmacological properties which are useful for analyzing specific mechanism of drug resistance. It can also predict the important pharmacokinetic parameters such absorption and metabolism by measuring drug concentration along different half lives. Furthermore, it is impossible to study every possible combination of drug that may be used clinically and to know their interaction. Therefore, the in vitro kinetic systems are being relied on screen and assess the antifungal drug interactions on yeast.

The first in vitro Dynamic Mycotic Infection model was evaluated the pharmacodynamic activities of fluconazole and amphotericin B given alone and in combination against *Candida albicans* (Lewis et al, 1998) (figure 1.19).

Because the majority of studies failed to detect polyene-azole antagonism utilized simultaneous administration of the azole antifungal and amphotericin B, the above study tried to detect this reaction and they showed that the rapidly fungicidal activity of amphotericin B may preempt the development of fluconazole-induced antagonism in yeast. Furthermore, they improved that the traditional methods for screening antifungal antagonism which rely only on static concentrations of antifungal agents introduced simultaneously which may be inappropriate for detecting polyene-azole antagonism.

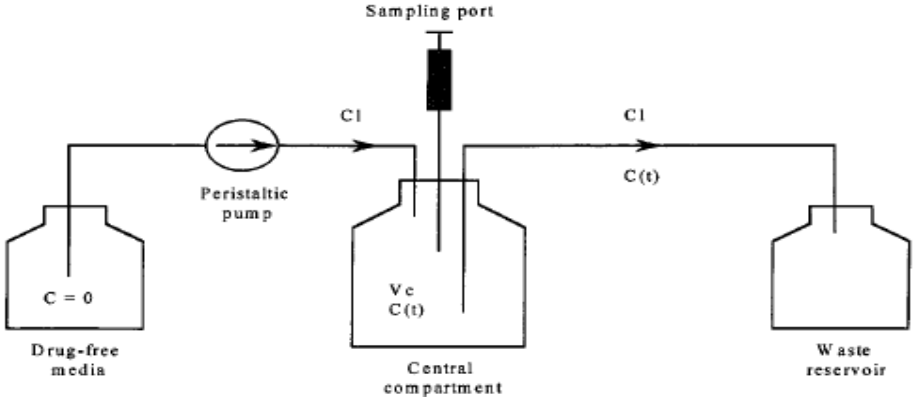

Figure 1.19. Schematic representation of a one-compartment in vitro infection model. Abbreviations: C, concentration of drug; Cl, flow rate; Vc, volume of the central compartment; C (t), concentration of drug in the central compartment (Lewis et al, 1998).

After that, Lignell and his colleagues (2007) were to develop and validate a new in-vitro kinetic model for the combination of two antifungal drugs with different half-lives, and to use this model for the study of the pharmacodynamic effects of amphotericin B and voriconazole, alone or in combination, against a strain of *Candida albicans* which was more reliable model with computer-controlled dosing pump to compensate the agent with the longer half-life. They showed also the antagonistic effect of voriconazole on the fungicidal activity of amphotericin B. But they advised to do further investigation to detect these effects according to species and doses.

Also Venisse et al (2008) did a mechanism-Based Pharmacokinetic/ Pharmacodaynamic Models of in vitro the fungistatic activity of fluconazole and the fungicidal activity of caspofungin effects against *Candida albicans*. They mproved their data with good fit of pharmacokinetic/ pharmacodaynamic parameters, especially with drug concentrations producing 50% of the maximal effect: 50% inhibitory concentrations for fluconazole growth inhibition and 50% effective concentrations for caspofungin death stimulation. The model has allowed the description of Candida growth inhibition by fluconazole and Candida death stimulation by caspofungin according to the documented mechanisms of action of these drugs. The used pharmacodaynamic parameter could be more useful in in vitro to compare drug potencies and to study drug combinations during preclinical and clinical development. Furthermore, the models has given much better characterization of drug efficacy over time than the MIC-based PK-PD index and could therefore represent an interesting alternative to optimize drug therapy during fungal infections by improving their efficacy.

1.12. The Aim of the PhD Thesis

The principal aim of this PhD thesis was to develop for the first time an in vitro model that reproducibly simulates the pharmacokinetics of amphotericin B, voriconazole, and caspofungin in humans after the intravenous administration of standard doses and to study the effects of these antifungal agents on clinical isolates of *A. fumigatus, A. flavus,* and *A. terreus* which represent the clinically most frequently encountered species.

After the establishment of this new in vitro model, the following issues were addressed:

- The effect of simulated human pharmacokinetics of increasing voriconazole dosages against clinical isolates A. fumigatus, A. flavus and A. terreus isolates with voriconazole MICs of 0.5 mg/l was examined; the magnitude of PKPD parameters associated with maximal activity was determined for each species.
- In vitro PKPD data of voriconazole were bridged with previously obtained human PK data in order to assess the efficacy of clinically administered voriconazole dosages against these life-threatening infections.
- The efficacy of amphotericin B against *A. fumigatus, A. flavus* and *A. terreus* was investigated using the new in vitro pharmacokinetic/pharmacodynamic model by simulating single-dose pharmacokinetics of amphotericin B in humans and monitoring Aspergillus growth over time based on the galactomannan production.
- The effect of simulated human pharmacokinetics of increasing Caspofungin dosages against clinical isolates *A. fumigatus, A. flavus* and *A. terreus* isolates.
- The efficacy of the combination of voriconazole with amphotericin B may be beneficial against *A. fumigatus* was assessed.

2.1. MATERIAL AND METHODS

2.1.1. Isolates

Three clinical isolates of *A. fumigatus*, *A. flavus*, and *A. terreus* obtained from patients with invasive pulmonary aspergillosis were used. The MICs of amphotericin B and voriconazole were 1 mg/liter and 0.5 mg/liter, respectively whereas the MEC of caspofungin was 0.5 mg/liter for all three isolates tested in triplicate with the CLSI M38A2 reference method (CLSI, 2008). The isolates were maintained at -70°C in 10% glycerol and were subcultured twice in Sabouraud dextrose agar at 30°C for 5 to 7 days before testing. Conidial suspensions were prepared in normal saline with 1% Tween 20. The inoculum concentration was determined with a Neubauer chamber and verified by culturing serial dilutions on Sabouraud dextrose agar.

2.1.2. Antifungal drugs.

Clinical intravenous formulations of voriconazole (Vfend; MW=0.35 kDa; Pfizer, Athens, Greece), amphotericin B (Fungizone; MW=0.92 kDa; Bristol-Myers, Athens, Greece), and caspofungin (Cancidas; MW=1.1 kDa; Merck, Athens, Greece) were reconstituted at 5000 mg/liter, 10000 mg/liter, and 5000 mg/liter, respectively, according to the manufacturers' instructions and stored at -70°C. Preliminary studies with pure drugs did not show any differences in fungal growth inhibition compared to clinical formulations. Therefore, all further studies were performed with clinical formulations.

2.1.3. Medium.

The medium used throughout this PhD thesis contained 10.4 g/liter RPMI 1640 with glutamine without sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), 0.165 M morpholinepropanesulfonic acid buffer (Invitrogen, Carlsbad, CA) and 100 mg/liter chloramphenicol (Sigma-Aldrich, St. Louis, MO) adjusted to pH 7.0 and filtered through 0.45μm pore size filter membrane.

2.1.4. In vitro pharmacokinetic/pharmacodynamic model.

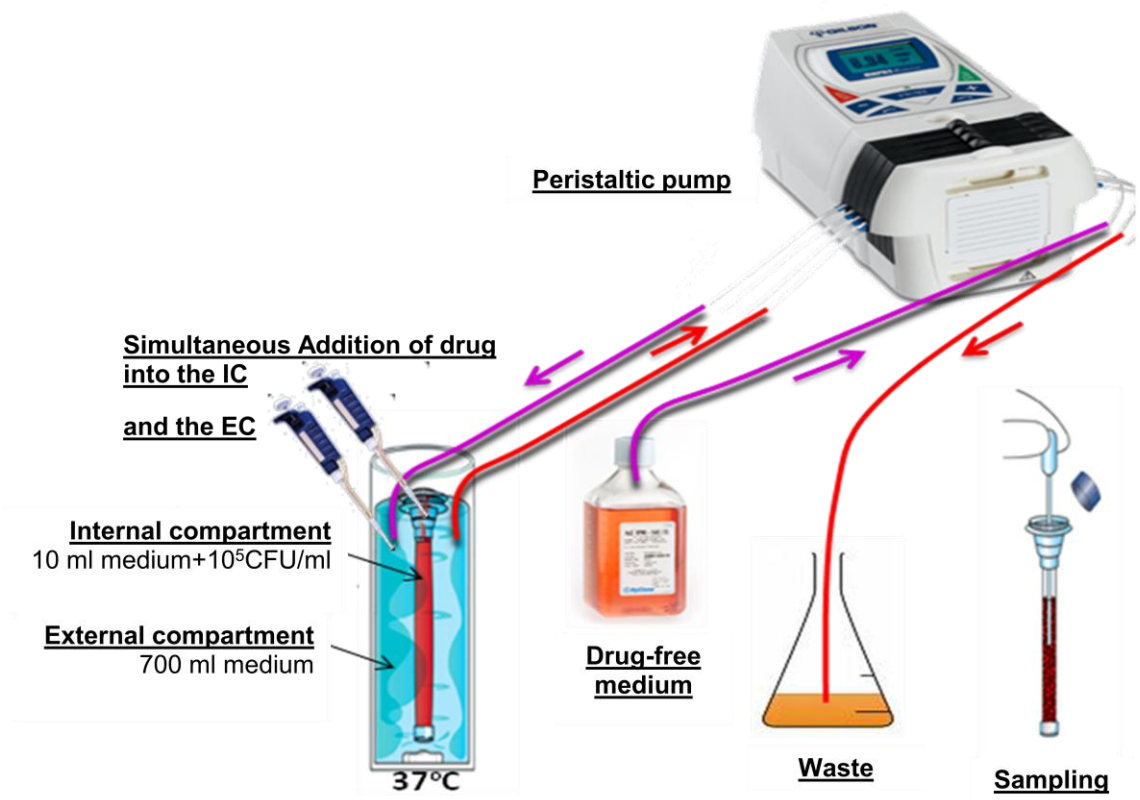

Figure 2.1. *In vitro* pharmacokinetic/pharmacodynamic model that simulated the pharmacokinetics of antifungal drugs in human plasma and enabled the study of their effects against *Aspergillus*.

The *in vitro* PK/PD simulation model developed in this PhD thesis is shown in figure 2.1. It consists of a 10-ml-volume dialysis tube (internal compartment IC) made of a semipermeable cellulose membrane (Float-A-Lyzer; Spectrum Europe B.V., Breda, The Netherlands) allowing the free diffusion of small molecules with MW<20 kDa. This was placed in a glass beaker containing 700 ml medium (external compartment EC) the content of which was continuously diluted by a peristaltic pump (Minipuls Evolution; Gilson Inc., Villiers le Bel, France) removing drug-containing medium from the EC and adding drug-free medium to the EC at a rate equivalent to the drug clearance in human plasma. At time zero, the IC was inoculated with 10 ml medium containing $1x10^5$ CFU/ml of *Aspergillus* conidia. The cellulose membrane of the IC allowed the free diffusion of nutrients and drugs until an equilibrium with the EC was reached, while at the same time it retained the conidia, hyphae, and macromolecular products such as galactomannan

(MW 25-75 kDa). Thus, galactomannan was concentrated inside the IC and was used as a biomarker of fungal growth. In order to ensure that galactomannan did not diffuse into the EC, galactomannan levels were also determined in the EC.

At time zero, the drug was simultaneously injected into the EC and the IC in order to achieve rapid equilibration of the drug concentrations in the two compartments; preliminary experiments showed that when the drug was added only to the EC there was a lag of 4 h until equilibrium was reached. The drug-containing medium in the EC was then continuously diluted with drug-free medium by the peristaltic pump adjusted to a specific flow rate in order to reproduce average drug half-lives ($t_{1/2}$s) observed in human plasma after the intravenous administration of amphotericin B (19 h), caspofungin (12 h), and voriconazole (6 h) in accordance with previous clinical studies (Ayestarán et al. 1996; Purkins et al. 2003; Walsh et al. 2005). The EC was then placed on a heated magnetic stirrer adjusted to 37°C and 2 rpm. The temperature and flow rate were checked regularly throughout the experiment using a thermometer and by measuring the volume of medium pumped out of the EC in 1 min, respectively.

2.1.5. Bioassays for determination of drug levels.

The drug levels in the EC and the IC were determined by microbiological methods using a *Candida kefyr* strain NCPF 3234 for voriconazole and caspofungin and a *Paecilomyces variotii* strain ATCC 22319 for amphotericin B as described previously for amphotericin B and voriconazole (Perea et al. 2000; Shadomy et al, 1969). Briefly, 3 x 10^5 CFU/ml *C. kefyr* or 5 x 10^5 CFU/ml *P. variotii* were inoculated into prewarmed medium (54°C) containing 15 g/liter agar (Difco Bacto Agar; BD Hellas SA, Athens, Greece). The medium was poured into square (10 by 10 cm) plastic Petri dishes, and after solidification, 2-mm-diameter holes were opened. One-hundred-microliter serial 2-fold drug dilutions (range, 0.25 to 16 mg/liter) and 100-μl samples obtained from the EC or IC medium were added to the holes. The plates were incubated at 37°C for 24 h, and the diameters of the inhibition zones around the holes were measured with a ruler. A standard curve of drug concentrations versus diameters of inhibition zones was constructed and subjected to linear regression analysis from known dilutions of drugs (range 0.25-16 mg/L). Based on this standard curve, the drug concentrations of the EC and the IC were determined at any time point. In order to ensure that the drug concentration within the IC was uniform,

samples from the center and periphery of the top, middle, and bottom parts of the IC were tested.

2.1.6. Pharmacokinetic analysis.

The following human doses of antifungal agents were simulated in the *in vitro* model: 3, 4, 5 mg/kg for voriconazole, 0.25, 0.5, 1, 1.5 mg/kg for amphotericin B, and 0.5, 1, 2 mg/kg for caspofungin, with steady state average peak total concentrations in human plasma of 1.7, 3.5, 7 ; 0.6, 1.2, 2.4, 4.8; and 5, 10, 20; mg/liter and mean residence time of 20-24h h for amphotericin B with triphasic plasma concentration profile, and average $t_{1/2}$s of 6 h for voriconazole and 12 h for caspofungin after intravenous administration (Bekersky et al. 2002, Ayestarán et al. 1996; Purkins et al. 2003; Walsh et al. 2005).

The levels of antifungal drugs at 0, 4, 6, 8, 20, and 24 h after the introduction of the drug were determined in the IC and EC with the bioassays. The data were subjected to non-linear regression analysis based on the one-compartment pharmacokinetic model, which is described by the equation $C_t = C_0 e^{-k/t}$, where C_t (dependent variable) is the drug concentration at a given time (independent variable), C_0 the initial drug concentration at 0 h, e is the physical constant 2.18, and k is the rate of drug removal. In addition, for amphotericin B, a three compartment model was also used to described the triphasic time-concentration profile based on the equation $C_t = C_a e^{-ka/t} + C_b e^{-kb/t} + C_c e^{-kc/t}$ where C_a, C_b and C_c and k_a, k_b and k_c are the maximum concentrations and rate constants for each of the three phases, respectively. The $t_{1/2}$ was calculated using the equation $t_{1/2}=k/0.693$ for the EC and the IC separately and compared with the respective values obtained in human plasma (Ayestarán et al. 1996; Purkins et al. 2003; Walsh et al. 2005) using Student's t test. For amphotericin B, $t_{1/2a}$, $t_{1/2,b}$ and $t_{1/2,c}$ was calculated for each phase. Finally, the area under the concentration-time curve from 0 to 24 h after drug administration (AUC_{0-24}) was calculated by the trapezoidal rule for each dose.

2.1.7. Galactomannan levels for determination of fungal growth.

Fungal growth in the IC was monitored by galactomannan production. Galactomannan levels were measured by enzyme-linked immunosorbent assay (Platelia; Bio-Rad Laboratories, Athens, Greece), and results were expressed as a galactomannan

index (GI) according to the manufacturer's instructions. In order to correlate GIs with fungal growth, different IC tubes were inoculated with 10^3, 10^4, 10^5, or 10^6 CFU/ml of *A. fumigatus* and incubated without a drug for 24 h at 37°C in the *in vitro* PKPD model, and GIs were determined at regular time intervals. The kinetics of the GIs were subjected to nonlinear regression analysis based on the Emax model described by the equation $E = E_{max} * T^{\gamma} / (T^{\gamma} + T_{50})$ where E is a GI (dependent variable), E_{max}, the maximum GI, T is the time (independent variable), T_{50} is the time corresponding to 50% of E_{max} and γ is the slope of the curve. The T_{50} parameter was then correlated with the initial concentration of the suspension of conidia by linear regression analysis.

1.2.8. Real-time PCR

Galactomannan levels were also correlated with real-time PCR results. IC tubes were inoculated with increasing inocula of *A. fumigatus*, and DNA was extracted from 100-µl samples with a Maxwell 16-cell DNA purification kit according to the manufacturer's instructions (Promega, Madison, WI) after incubation for 2, 4, 8, and 24 h at 37°C in the *in vitro* PK/PD model. Real-time PCR (species specific) was performed with the MycAssay Aspergillus kit according to the manufacturer's instructions (Mycognostica, Manchester, United Kingdom). The threshold cycle (CT) of each sample, which identifies the cycle number during PCR when fluorescence exceeds a threshold value determined by the software, was converted to conidial equivalent (CE) A. fumigatus DNA by interpolation from a 4-point standard curve of CT values obtained with 10^3 to 10^6 Aspergillus fumigatus CFU/ml. The area under the GI-versus-time curve was then correlated with the area under the PCR CE-versus-time curve by linear regression analysis.

2.1.9. Pharmacodynamic analysis.

The GIs in the IC inoculated with 10^5 CFU/ml *A. fumigatus*, *A. flavus*, or *A. terreus* were measured at regular time intervals up to 72 h. The pharmacodynamic data were subjected to nonlinear regression analysis based on the E_{max} model as described above. The goodness of fit of the E_{max} model was assessed by R^2, analysis of the residuals and visual inspection of the curves. The parameters T_{50} and E_{max} of GI-versus-time curves in the presence of drugs ($T_{50,D}$ and $E_{max,D}$) were compared with the corresponding

parameters of drug-free growth controls ($T_{50,GC}$ and $E_{max,GC}$). The percent reduction was calculated as 100% - ($E_{max,D}$-$E_{max,GC}$)/$E_{max,GC}$ and the difference $T_{50,GC}$-$T_{50,D}$ was calculated for each drug and species. All the experiments were repeated at least twice.

2.1.10. Combination of voriconazole with amphotericin B against *Aspergillus fumigatus*

Drug combination dosages of amphotericin B and voriconazole were simulated in the in vitro PKPD model with a half-life of 19h and 6h, respectively. The flow rate of the peristaltic pump was adjusted to simulate the drug with shortest half-life and the drug with the longest half-life was supplemented in order to compensate the loss because of the faster elimination of the first drug. Twelve different combination regimens were investigated with C_{max} 0.1, 0.6, 1.2 and 2.4 of amphotericin B and 1.75, 3.5 and 7 mg/l of voriconazole including single-drug and drug-free regimens. The IC was inoculated with a conidial suspension (10^3 CFU/mL), while drug solutions were injected in both compartments every 12h for voriconazole and every 24h for amphotericin B simulating human dosing regimes. Drug levels were determined with bioassays and fungal growth based on galactomannan concentrations in the IC with the sandwich-ELISA described above. The PD analysis for the interactions studied was described using the Bliss independence model.

2.1.11. Bridging in vitro PKPD data with human PK

In vitro PKPD data were bridged to human PK data combining in vitro AUC_{GI}/$fAUC_{0-12}$ and clinical $fAUC_{0-12}$ taking into account the inter-patient variation. The % target attainment of the upper and lower 95% confidence interval limits of voriconazole $fAUC_{0-12}$s observed previously in patients were calculated based on the in vitro AUC_{GI}/$fAUC_{0-12}$ relationship for each species.

2.1.12. Bliss independence intraction analysis

Bliss independence is described by the equation $I_{IND}=I_A+ I_B - I_A \times I_B$ for a certain combination of x mg/L of drug A and y mg/L of drug B where I_A: % fungal growth inhibition at x mg/L of drug A alone, I_B: % fungal growth inhibition at y mg/L of drug B

alone and I_{IND}: expected % fungal growth inhibition of a noninteractive (independent) theoretical combination of x mg/L of drug A with y mg/L of drug B. Because, % growth inhibition I equals to 1-E where E if % fungal growth Bliss equation can be transformed to $E_{IND}=E_A \times E_B$ where E_A and E_B are % of fungal growth of each drug A and B, respectively.

The difference ($\Delta I=E_{IND} - E_{OBS}$) between the expected % fungal growth E_{IND}, and the experimentally observed % fungal growth E_{OBS}, describes the interaction of each combination of the concentrations of the two drugs. If ΔE is >0 ($E_{OBS} < E_{IND}$, and hence, less growth was observed than if the two drugs were acting independently), Bliss synergy is concluded for that particular combination. If ΔE is <0 ($E_{OBS} > E_{IND}$, and hence, more growth was observed than if the two drugs were acting independently), Bliss antagonism is concluded for that particular combination. In any other case, the conclusion is Bliss independence. For each combination of x mg/L of AMB with y mg/L of VOR the ΔE was calculated, its statistical significance was assessed by Student's t test and the interaction was assessed as described above.

All of the experiments were repeated at least twice. All statistical analysis was performed with the software Prism 5.01 (GraphPad Inc., La Jolla, CA).

2.2. RESULTS

2.2.1 Bioassays for determination of antifungal drug concentrations

The standard curves of the diameter of inhibition zone diameters versus drug concentrations obtained with the bioassays are shown in Figure 2.2 for each antifungal drug. The drug concentrations determined with the bioassay ranged from 0.5 mg/liter to 16 mg/liter. The diameter of the inhibition zone correlated linearly with the $\log_{10}$ drug concentration (r^2 > 0.77). The intra- and inter-experimental coefficients of variation ranged from 5% at low drug concentrations to 15% at high drug concentrations, with an average of 8%.

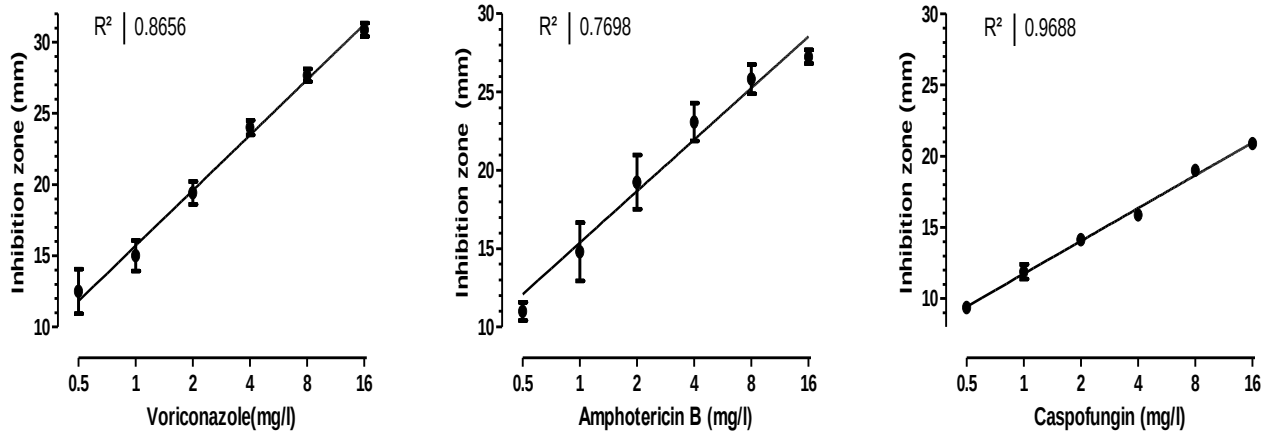

Figure 2.2. Microbiological methods for determining levels of amphotericin B, voriconazole and caspofungin. Graphs show diameter of inhibition versus drug concentration and the correlation coefficient. The intra-and inter-experimental variation was <10%.

2.2.2. Galactomannan production as a marker of fungal growth

The production of galactomannan by *A. fumigatus* followed a sigmoid curve described very well by the E_{max} model (r^2 >0.98) (Figure 2.3). The parameter T_{50} was inversely associated with the initial concentration of conidia (r^2> 0.98; slope, -4.4 ± 0.4) and was therefore considered valid for quantifying fungal growth. Real-time PCR of *Aspergillus* DNA confirmed that the total increase in PCR CEs was associated with the total increase in galactomannan over time as assessed with the areas under the corresponding curves (r^2> 0.98) (Figure 2.4).

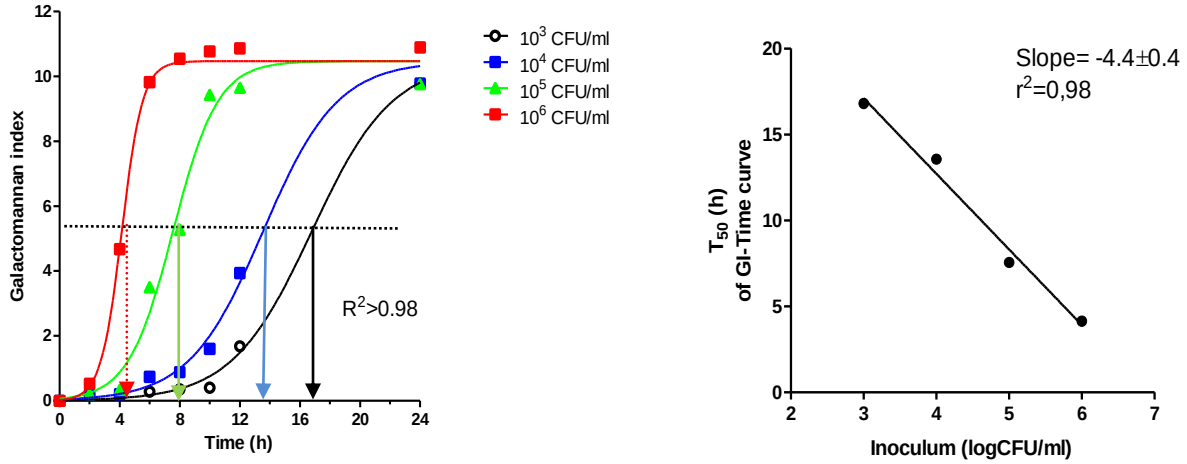

Figure 2.3. Kinetics of galactomannan productions of different inocula of A. fumigatus (left graph) and correlation parameter of the T_{50} E_{max} model with the concentration of nits in the suspension (right graph). The intermittent arrows on the left graph showed the parameters of T_{50} (the time corresponding to 50% of E_{max}) for each curve.

Although we did not assess the validity of using galactomannan to estimate the fungal burdens of other species/strains, any species/strain-dependent difference in galactomannan production will affect both drug treated and drug-free control samples, hence without altering the net effect.

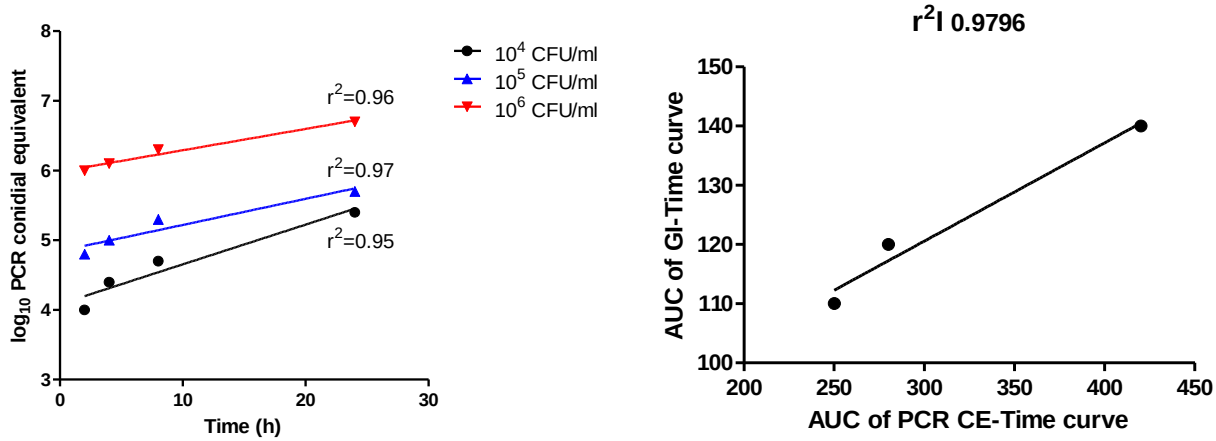

Figure 2.4. Kinetics of real-time PCR CEs for three increasing inocula of *A. fumigatus* (left graph) and correlation between the area under the PCR CE-versus-time curve and the area under the GI-versus-time curve as in Fig. 2.3 (right graph). Results from experiments with a starting inoculum of 10^3 CFU/ml were excluded from the analysis because the PCR signal was very close to the lower limit of detection, resulting in highly variable results.

2.2.3. Pharmacokinetic analysis

The drug concentration-time profiles of the three antifungal drugs (voriconazole, amphotericin B and caspofungin) in both the EC and the IC corresponded to those

previously observed in human plasma (Ayestarán et al. 1996; Purkins et al. 2003; Walsh et al. 2005). For all antifungal drugs, the AUC_{0-24} values in the *in vitro* model were similar to the corresponding measurements in humans (Table 2.1).

Table 2.1. Pharmacokinetic parameters for amphotericin B, caspofungin and voriconazole in humans and in vitro pharmacokinetic / pharmacodynamic system.

Drug (human dose)	Pharmacokinetics parameters[a]	Human plasma (mean values)[b]	In vitro system (mean±SEM)[c]
Amphotericin B	**C_{max} (mg/l)**	2.83	2.6±0.1
(1 mg/kg)	**$t_{1/2}$ (h)**	19.65	11±1.5[d]
	AUC_{0-24} (mg.h/l)	28.98	34.52
Voriconazole	**C_{max} (mg/l)**	3.62	3.7±0.17
(4 mg/kg)	**$t_{1/2}$ (h)**	6.5	5.9±0.6
	AUC_{0-24} (mg.h/l)	22.7	30.37
Caspofungin	**C_{max} (mg/l)**	10	9.3±0.25
(1 mg/kg)	**$t_{1/2}$ (h)**	12.2	14±1.25
	AUC_{0-24} (mg.h/l)	97.20	120.31

[a]Cmax: maximum concentration, t1/2: half-life, AUC0-24: area under the concentration-time curve 0 to 24h after the dose
[b]Data derived from previous clinical studies (Ayestaran, 1996;Purkins, 2003;Walsh, 2005)
[c]IC: internal compartment (mean±SEM)
[d]based on one-compartment model

2.2.3.1 Pharmacokinetics of Voriconazole

The one compartment pharmacokinetic model described well the drug levels in the IC (R^2>0.97). Intra-and inter-experimental variation of the in vitro PK data was <10 % (Figure 2.5). In vitro voriconazole pharmacokinetics were close to the target values observed in human plasma after administration of 3, 4 and 5 mg/kg voriconazole (Purkins et al, 2002, Purkins et al, 2003). The $t_{1/2}$ of voriconazole in the in vitro system was 5.7-6.5h, which was similar to the half-life of 4.7-7.3h observed in patients' plasma. The voriconazole AUC_{0-12} was 11.6, 23.5 and 43.8 mg.h/l for the three doses; likewise, in human plasma, the respective values for voriconazole dosages 3, 4 and 5 mg/kg corresponded to 5.8, 12.4 and 18.2 mg.h/l (calculated based on 58% protein binding and 13.9, 29.5 and 43.4 mg.h/l total AUC_{0-12})(Purkins et al, 2002, Purkins et al, 2003).

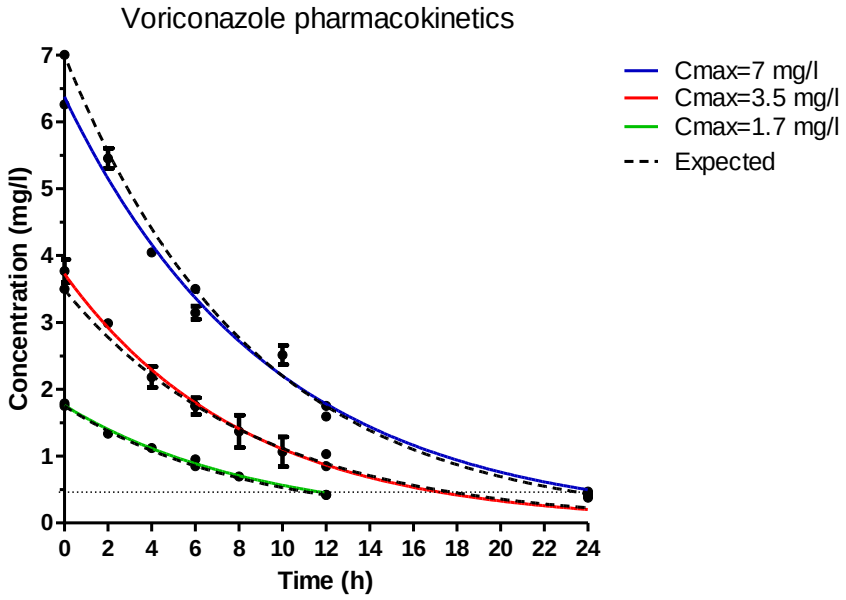

Figure 2.5. Pharmacokinetic analysis of simulated doses 3, 4 and 5 mg/kg voriconazole in humans and in vitro PKPD model with C_{max} 1.7, 3.5 and 7 mg/l, respectively and half-life 6h.

2.2.3.2 Pharmacokinetics of Amphotericin B

The C_{max} in the IC were 0.76-0.78, 1.05-1.10, 2.5-2.7 and 3.9-4.4 mg/l and AUCs of 4.5-5, 8-8.6, 31.9-33.2, 64.8-67.9 mg.h/l, respectively with $t_{1/2,\alpha}$ 0.2-2h, $t_{1/2,\beta}$ 10-17h and $t_{1/2,\gamma}$ 71h for the simulated amphotericin B doses 0.25, 0.5, 1 and 1.5 mg/kg, respectively. Because of the low limit of detection with the bioassay, the gamma phase was observed only for the highest dose of amphotericin B with C_{max} 4.8 mg/l. These values were similar to those observed in human plasma after administration of amphotericin B doses 0.25-1.5 mg/kg with the largest deviations observed at lower doses (Ayestarán et al. 1996) (Figure 2.6).

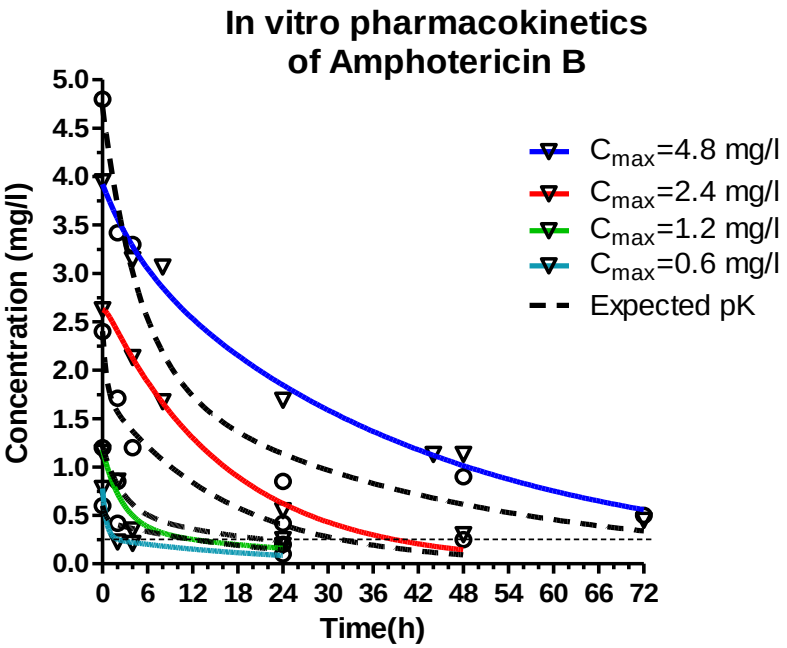

Figure 2.6. Pharmacokinetic analysis of simulated doses 0.3, 0.5, 1 and 2 mg/kg amphotericin B in humans and in vitro PKPD model with C_{max} 0.6, 1.2, 2.4 and 4.8 mg/l, respectively and half-life 19h.

2.2.3.3 Pharmacokinetics of Caspofungin

The one compartment pharmacokinetic model described well the drug levels in the IC (R^2>0.968). Intra-and inter-experimental variation of the in vitro PK data was <10 % (Figure 2.7). In vitro caspofungin pharmacokinetics were close to the target values observed in human plasma after administration of 0.5, 1 and 2 mg/kg caspofungin (Walsh et al. 2005). The $t_{1/2}$ of caspofungin in the in vitro system was 11.6-14.8h, which was similar to the half-life of 12.2h observed in patients' plasma (Walsh et al. 2005).

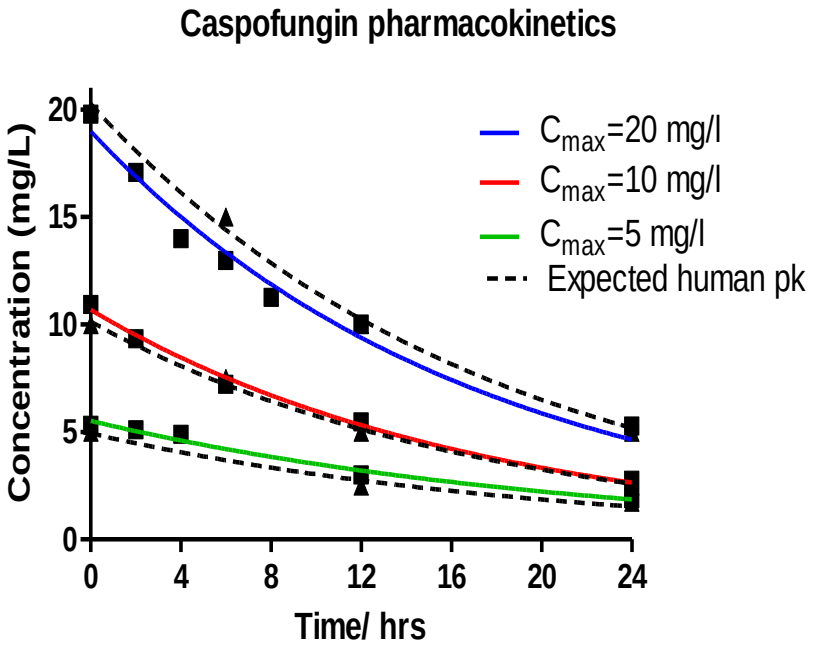

Figure 2.7. Pharmacokinetic analysis of simulated doses 0.5, 1 and 2 mg/kg caspofungin in humans and in vitro pharmacokinetic/pharmaco dynamic system with C_{max} 5, 10 and 20 mg/l, respectively and half-life 12h.

2.2.4. Pharmacodynamic analysis

The pharmacodynamic data from the simulated doses of antifungal agents against the three isolates showed that the E_{max} model described the data well, as demonstrated by an R^2 value of >0.86 and the normal distribution of residuals around 0. After 72 h of incubation, the E_{max} and T_{50} parameters for the standard doses of three antifungal agents (voriconazole, amphotericin B and caspofungin) against three *Aspergillus* species are shown in (Table 2.2).

2.2.4.1 Pharmacodynamics of voriconazole

The pharmacodynamic data for each simulated voriconazole dose against the three strains are shown in Figure 2.8. The E_{max} model described the data well (R^2> 0.86).

Table 2.2 Pharmacodynamic parameters mean±standard error of simulated doses of amphotericin B (AMB), voriconazole (VOR), and caspofungin (CAS) against *A. fumigatus*, *A. flavus*, and *A. terreus* isolates in the *in vitro* PKPD model.

Drug (Dose)	Species	E_{max} (GI)			$T_{1/2}$ (h)		
		- drug	+ drug	decrease	- drug	+ drug	difference
AMB	*A. fumigatus*	10.2±0.6	0	-100%*	5.3±0.7	>72h	>-66.7*
1 mg/kg	*A. flavus*	11.5±0.2	11.12±0.3	-3.6%	7.5±0.6	30.31±4.2	-22.8*
	A. terreus	10.7±0.4	10.96±1.7	2.2%	4.45±0.6	11.98±2.8	-7.53*
VOR	*A. fumigatus*	11.2±0.2	5.70±0.1	-49.5%*	14.81±0.6	18.80±0.3	-3.99*
4 mg/kg	*A. flavus*	11.3±0.4	10.63±0.1	-6.3%	7.13±0.7	3.82±0.1	3.31*
	A. terreus	12.0±0.3	8.66±0.3	-27.9%*	10.92±0.9	16.29±1.3	-5.37*
CAS	*A. fumigatus*	9.2±0.1	8.97±0.1	-2.9%	8.28±0.3	8.22±0.1	-0.02
1 mg/kg	*A. flavus*	9.3±0.1	9.21±0.1	-1.8%	3.26±2.1	4.41±0.5	1.15
	A. terreus	9.2±0.4	8.83±0.1	-4.7%	7.68±0.5	10.48±0.5	2.8

* Statistically significant ($p<0.05$)

The three simulated voriconazole doses resulted in different GI-time curves in terms of the extent and rate of galactomannan production as reflected by the different E_{max}, T_{50} and γ parameters of the E_{max} model.

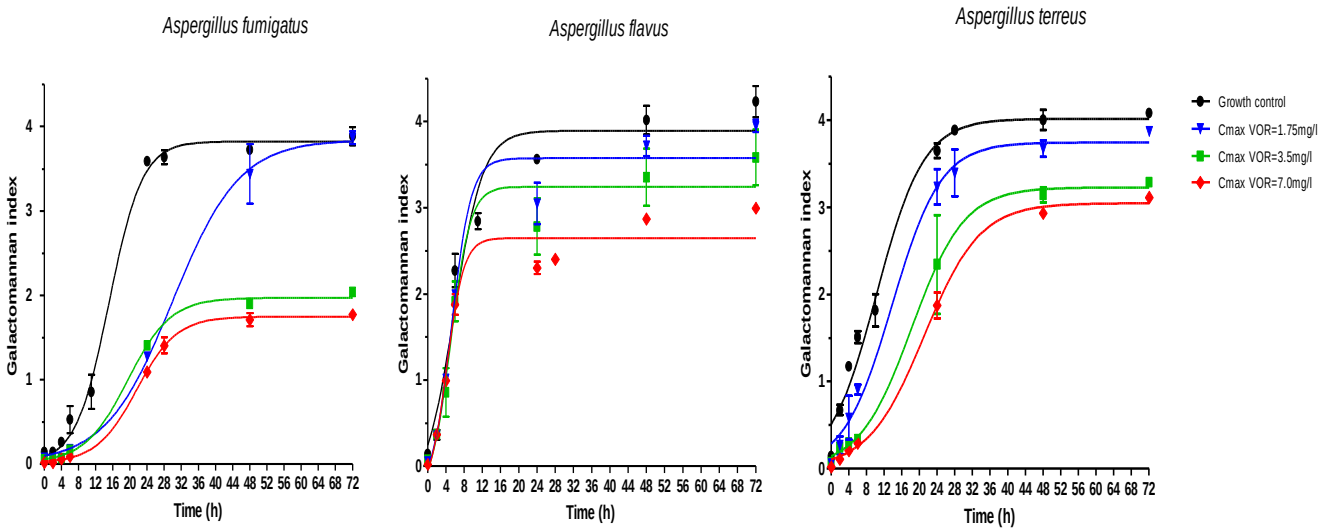

Figure 2.8. Pharmacodynamic analysis of simulated doses 3, 4 and 5 mg/kg of voriconazole against A. fumigatus (left graph), A. flavus (middle graph) and A. terreus (right graph) isolates based on galactomannan index in the in vitro PKPD model.

In particular, all three voriconazole doses delayed galactomannan production by *A. fumigatus* ($T_{50,GC}$=15h 204 vs. $T_{50,D}$=19-29h) whereas only the two highest doses decreased the maximum galactomannan production compared to growth control ($E_{max,}$GC=3.8 vs. $E_{max,}$1.75=3.8, $E_{max,}$3=2, $E_{max,}$7= 1.8). In the case of *A. flavus*, there were no significant differences in T_{50} parameters ($T_{50,GC}$=5.8h vs $T_{50,GC}$=4.2-5.3), whereas maximum galactomannan production was reduced as voriconazole dose increased ($E_{max,GC}$=3.9 vs. $E_{max,}$1.75=3.6, $E_{max,}$3.5=3.2, $E_{max,}$7=2.6). Regarding *A. terreus*, voriconazole delayed galactomannan production ($T_{50,GC}$=10.5h vs $T_{50,}$1.75=14h, $T_{50,}$3=18h, $T_{50,}$7=21h) whereas maximum galactomannan production was reduced as voriconazole dose increased ($E_{max,GC}$=4 vs. $E_{max,}$1.75=3.7, $E_{max,}$3.5=3.2, $E_{max,}$7=3).

2.2.4.1.1. Pharmacokinetic-pharmacodynamic analysis of voriconazole

In order to capture the above described changes in E_{max} model parameters of GI-time curves, the AUC_{GI}, which was used as surrogate marker of fungal growth, was correlated to the area under the concentration-time curve for each voriconazole dose ($fAUC_{0-24}$). The AUC_{GI} at 24h decreased from 34 GIxh in the growth control to 10.5 GIxh at the highest voriconazole dose for *A. fumigatus* following a sigmoid pattern (R^2>0.97) (Figure 2.9, left graph).

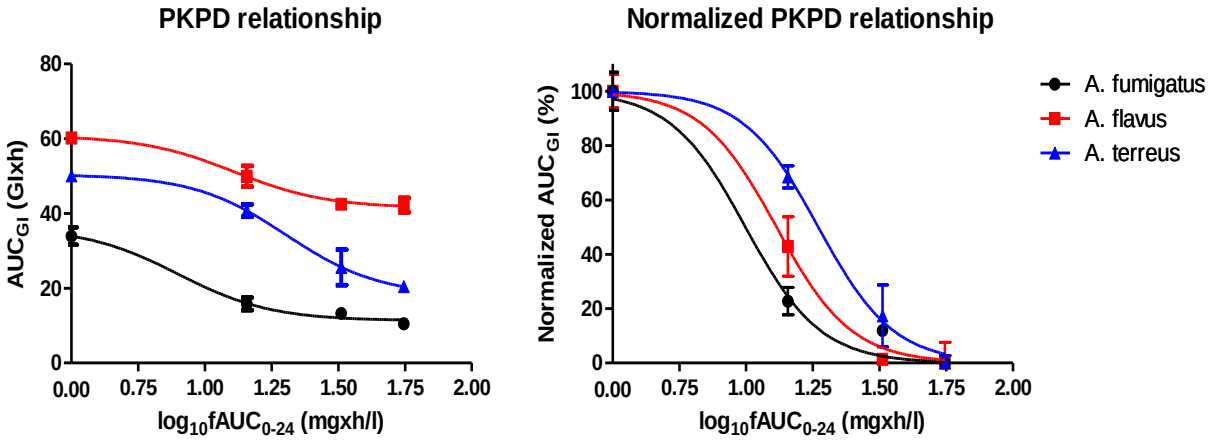

Figure 2.9. In vitro PKPD relationship of voriconazole. The relationship between the area under the galactomannan index curve (AUC_{GI}) (left graph) or normalized AUC_{GI} (right graph) and the area under the concentration time curve ($fAUC_{0-24}$) for each Aspergillus species. The AUC_{GI} for the first 24h was used as the surrogate marker of fungal growth.

The same pattern of AUC_{GI} reduction was observed for *A. flavus* (60.3 GIxh in growth control to 42.3 GIxh at the highest voriconazole dose) and for *A. terreus* (50.1 GIxh to 20.5 GIxh, respectively). Because of the different dynamic AUCGI ranges among the three *Aspergillus* spp., the AUC_{GI} was normalized from 0% to 100% based on the minimum and maximum AUC_{GI}, respectively (Figure 2.9, right graph). Based on the normalized PKPD relationship, the AUC_{0-24} associated with near-maximum activity (10% AUC_{GI}) was 18.9 (14.4-23.1) mg.h/l against *A. fumigatus*, 26.6 (21.1-32.9) mg.h/l against *A. flavus* and 36.2 (27.8-45.7) mg.h/l against *A. terreus* ($F_{2,19}$=17.22, p<0.0001). These AUC_{0-24}s were similar when the AUC_{GI} at 48h and 72h were used as markers of fungal growth. Of note, the AUC_{0-24} of 18.9 mg.h/l for the tested *A. fumigatus* isolate (MIC=0.5 mg/l) is close to the fAUC/MIC of 15.3 (based on 58% protein binding in mice) associated with 90% survival in an animal model of experimental aspergillosis (Mavridou, et al, 2010); thus providing a validation of the in vitro PKPD model.

2.2.4.1.2. Bridging the in vitro voriconazole PKPD data with human PK data

Low median % target attainment (<45%) was found for 3 mg/kg voriconazole dosing for all species (Table 2.3). The standard dosage of 4 mg/kg was associated with high median % target attainment (80%) for *A. fumigatus* but not for *A. flavus* and *A. terreus* for which as low as 17% and 6% target attainment, respectively, was found for $fAUC_{0-12}$ at the lower 95% confidence interval limit observed in patients. Even for *A. fumigatus* a wide range of % target attainment (32-97%) was detected reflecting the wide variation of voriconazole $fAUC_{0-12}$ among patients receiving 4 mg/kg. In agreement with these findings is the range of 50 to 80% survival rate reported in clinical trials among patients with aspergillosis treated with voriconazole. Importantly, for patients infected by *A. fumigatus* isolates with voriconazole MICs of 0.5 mg/l the 6-week survival rate was ~75% (Baddley et al, 2009), which is very close to the 80% median % target attainment recorded in the present study providing, thus, clinical validation of the in vitro model. With a dosage of 5 mg/kg more patients will attain the target for *A. fumigatus* infections (>86%) whereas >73% and >47% will achieve the target for *A. flavus* and *A. terreus* infections. Of note, the % of target attainment for the upper 95% confidence interval of 4 mg/kg voriconazole dosage was >83% for all *Aspergillus* species (Table 2.3).

Table 2.3. Predicted efficacy of voriconazole dosing regimens after bridging the in vitro drug exposure-efficacy relationship with human voriconazole exposure in plasma.

Dose (mg/kg)	Mean of (95% CI) $fAUC_{0-24}$ (mg*h/l) (Purkins et al, 2003)	Mean (95%CI) % target attainment		
		A. fumigatus	***A. terreus***	***A. flavus***
3	10,4(8,4-14,8)	24(11-45)	12(5-26)	4(2-11)
4	23,2(12-44)	80(32-97)	63(17-93)	36(6-83)
5	34,4(26,4-44)	93(86-97)	86(73-94)	68(47-83)

2.2.4.2. Pharmacodynamics of amphotericin B

The GI–time curves were described very well with the E_{max} model (R^2> 0.86) and they were characterized by the same E_{max} but different slopes and T_{50}s for the different amphotericin B doses and *Aspergillus* species. Among all species and doses tested, complete inhibition of galactomannan production was observed only against *A. fumigatus* with amphotericin B doses corresponding to C_{max} ≥2.4 mg/l. At lower doses, a significant delay in galactomannan production was observed with T_{50} of 35.8h for C_{max} 0.6 mg/l and 50.4h for C_{max} 1.2 mg/l compared to drug free control T_{50}=4.2h (p<0.001). For *A. flavus*, there was no complete inhibition but a progressive delay of galactomannan production with increasing amphotericin B doses since the T_{50} increased from 8h at the growth control to 9h at amphotericin B dose with C_{max} 0.6 mg/l, 21h at C_{max} 1.2 mg/l, 34h at C_{max} 2.4 mg/l and 55h at C_{max} 4.8 mg/l (p<0.05). For *A. terreus*, the delay in galactomannan production was modest since the T_{50} of the growth control increased from 4h to 10h at the highest dose of amphotericin B with C_{max} 4.8 mg/l (p>0.05)(Figure 2.10).

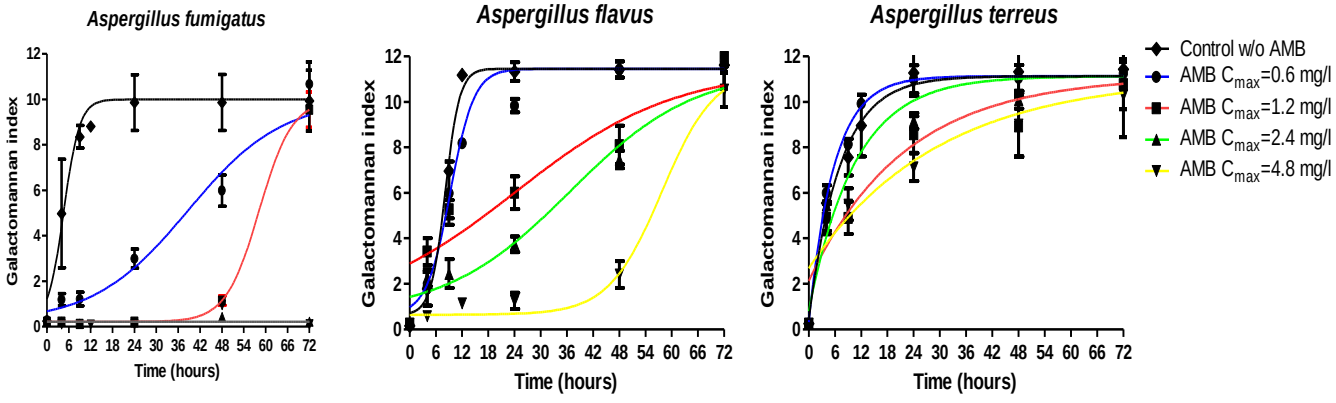

Figure 2.10. Single-dose pharmacodynamic analysis of simulated amphotericin B doses with C_{max} 0.6, 1.2, 2.4 and 4.8 mg/l against *A. fumigatus* (left graph), *A. flavus* (middle graph) and *A. terreus* (right graph) isolates based on galactomannan index in the in vitro pk/pd system.

Finally, the in vitro activity of amphotericin B against the three Aspergillus species was compared by constructing PK-PD curves. In order to quantify the effect of each amphotericin B dose at the entire 72h period of incubation, the AUC_{GI} was calculated for each dose and species as a surrogate marker of fungal growth and plotted against the corresponding C_{maxs}. The in vitro PK-PD relationship followed a sigmoid pattern (global R^2=0.99). The C_{max} associated with 50% activity for *A. fumigatus* was 0.60 with 95% confidence interval 0.49-0.72 and it was statistically significant lower that against *A. flavus* 3 (2.4-3.6) and *A. terreus* 5.9 (3.7-9.5) (p<0.001) (Figure 2.11).

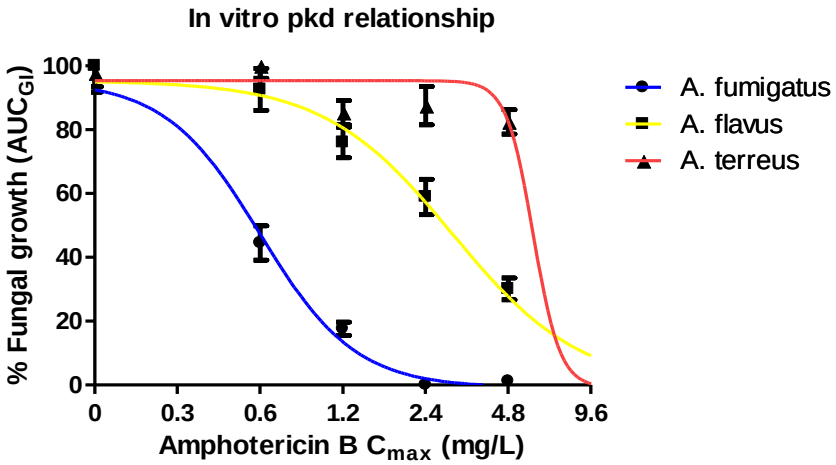

Figure 2.11. Single-dose exposure-efficacy relationships of amphotericin B against each *Aspergillus* species in the in the in vitro pk/pd system based on the increasing amphotericin B C_{max} (maximum concentration) and the galactomannan index.

2.2.4.3. Pharmacodynamics of caspofungin.

The GI–time curves were described very well with the E_{max} model (R^2> 0.86) and

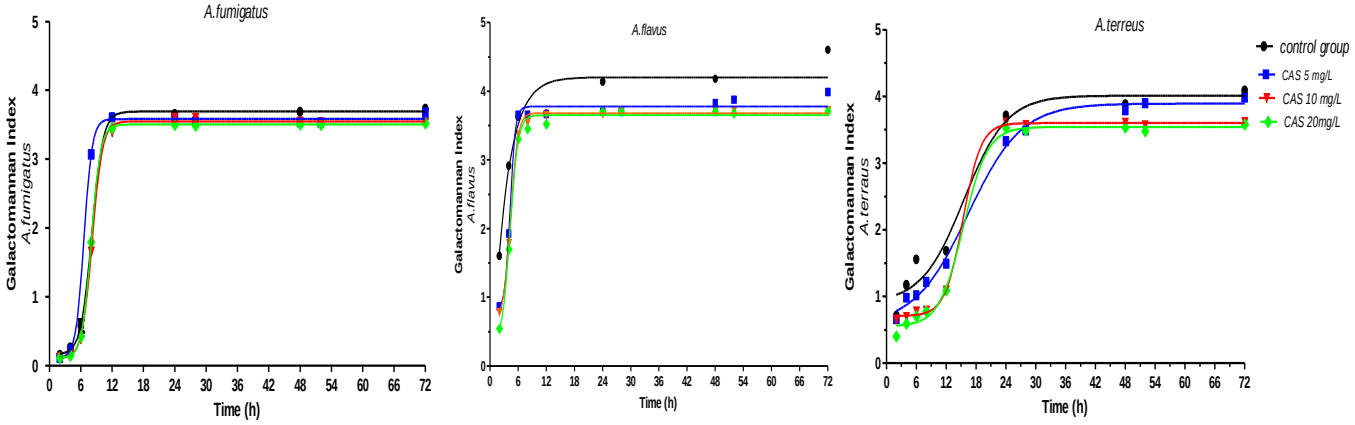

Figure 2.12. Single-dose pharmacodynamic analysis of simulated caspofungin doses with C_{max} 5, 10 and 20 mg/l against *A. fumigatus* (left graph), *A. flavus* (middle graph) and *A. terreus* (right graph) isolates based on galactomannan index in the in vitro pk/pd system.

they were characterized by the same E_{max} but different slopes and T_{50}s for the different caspofungin doses and *Aspergillus* species. Caspofungin did not have any effect on galactomannan production by *A. fumigatus* and *A. flavus*; whereas a delay of 2.8 h in galactomannan production was observed for *A. terreus* (figure 2.12).

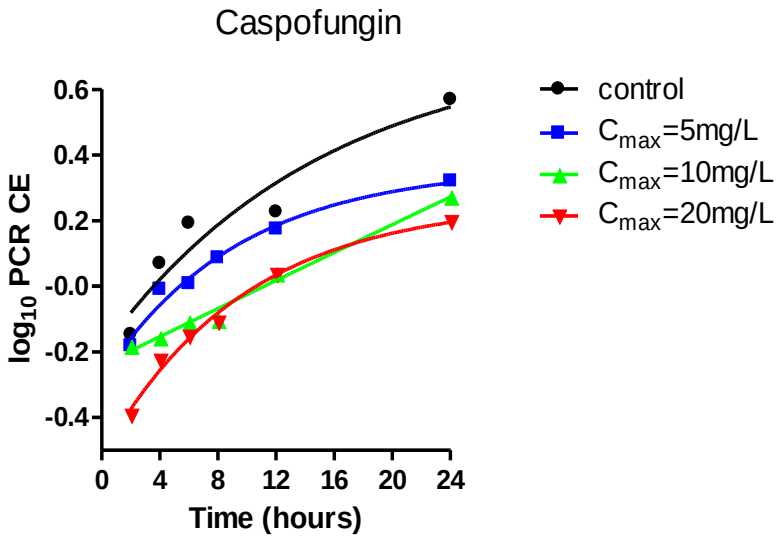

Figure 2.13. Real-time PCR of log growth reductions in the conidial equivalents (CE) of *A. fumigatus*, in the presence of caspofungin (C_{max}: 5, 10 and 20 mg/L respectively compared to those of the drug-free control.

When *Aspergillus* DNA was measured by real-time PCR, 2.2-, 0.9-, and 1.6-log reductions in the CEs of *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively, were observed after 6 h of incubation in the *in vitro* PK/PD model with (C_{max}=10 mg/L) and extend to 8h with (C_{max}=20 mg/L) in the presence of caspofungin compared to those of the drug-free controls (figure 2.13).

2.2.5. Voriconazole+amphotericin B combination against *A. fumigatus*

The pharmacokinetics of amphotericin B and voriconazole multidosing combination regimens every 24h and 12h respectively are shown in Figure 2.14 for one combination with C_{max} 2.4 mg/l and 1.7 mg/l, respectively. In vitro pharmacokinetics approximated human pharmacokinetics for both drugs and all dosages (data not shown).

Fungal growth was gradually reduced by exposure to higher drug concentrations in both monotherapies with complete inhibition observed at amphotericin B and voriconazole dosages with C_{max} ≥0.3 mg/l and ≥3.4mg/l.

The combination of voriconazole with amphotericin B exerted antagonistic effects (-4 to -13%) in the wider range of concentrations tested (VOR 1.7 mg/L with AMB 0.6, 0.3 and 0.1 mg/L), whereas some synergistic interactions at a percentage of 26% were observed at

lower amphotericin B concentrations (≤0.1 mg/L) as shown in Table 2.4. The synergistic combination is shown in Figure 2.15).

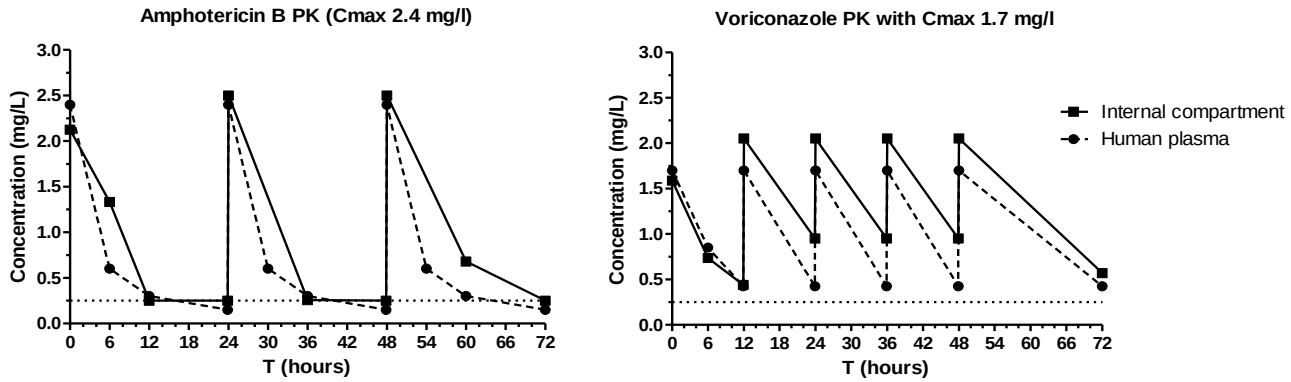

Figure 2.14. The pharmacokinetics of amphotericin B and voriconazole multidosing combination regimens every 24h and 12h respectively for one combination with C_{max} 2.4 mg/l and 1.7 mg/l, respectively.

Table 2.4. Bliss independence drug interaction analysis of voriconazole+amphotericin B combination in the in vitro PKPD model.

AMB	**VOR 1.7 mg/l**			**VOR 3.4 mg/l**			**VOR 7.2 mg/l**		
mg/l	E_{OBS}	E_{IND}	ΔE	E_{OBS}	E_{IND}	ΔE	E_{OBS}	E_{IND}	ΔE
2.4	5.9±1.3	1.46±0.32	-4.44	6.32±1.39	0.24±0.05	-6.08	4.3±0.95	0.23±0.05	-4.07
0.6	7.08±0.85	1.41±0.17	-5.67*	9.25±1.11	0.18±0.02	-9.07*	7.12±0.85	0.19±0.02	-6.93*
0.3	10.92±1.31	1.54±0.18	-9.38*	12.84±1.54	0.12±0.01	-12.72*	9.56±1.15	1.37±0.16	-8.19*
0.1	8.62±1.03	34.46±4.14	25.84*	16.31±1.96	3±0.36	-13.31*	10.11±1.21	2.79±0.33	-7.32*

*statistically significant based on t test

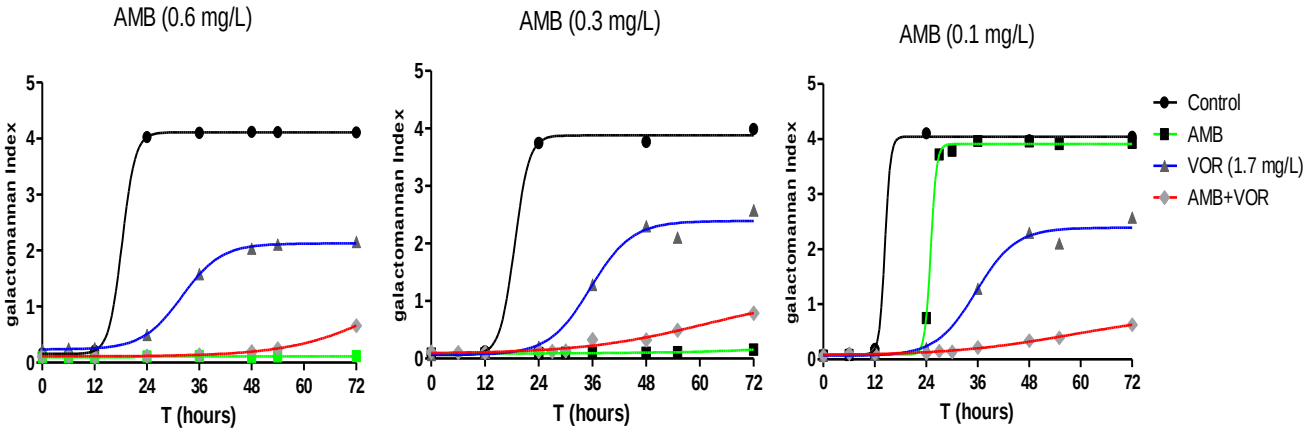

Figure 2.15. Pharmacodynamics of voriconazole in combination with low concentration of amphotericin B against *Aspergillus fumigatus* isolates (Left: VOR 1.7 mg/L + AMB 0.6, Middle: VOR 1.7 mg/L + 0.3 and Right: VOR 1.7 mg/L + 0.1 mg/L).

2.3.1. DISCUSSION

Here we report for the first time, to our knowledge, a new *in vitro* pharmacokinetic model for *Aspergillus* species that simulates the human plasma pharmacokinetics of three major antimould agents (voriconazole, amphotericin B and caspofungin) after the intravenous administration of standard doses (2, 3, 4, 5 mg/kg, 0.25, 0.5, 1, 2 mg/kg, and 0.5, 1, 2 mg/kg, respectively). For the pharmacodynamic analysis of these regimens, galactomannan production was determined as a surrogate marker of fungal growth.

Three *Aspergillus* strains representing the most clinically significant species (*A. fumigatus*, *A. flavus*, and *A. terreus*) were tested by this model. The isolate of each species was deliberately selected for identical MICs of a given antifungal drug. Thus, the MICs were the same for amphotericin B (1 mg/liter) and voriconazole (0.5 mg/liter) and the MECs were also the same (0.5 mg/liter) in order to elucidate the species-specific *in vitro* PK/PD effects of a given antifungal drug that were not necessarily reflected by the MIC.

The new in vitro system overcame the main disadvantage of constant concentration of antifungal agents when applied in conventional in vitro susceptibility methodologies (Lewis et al, 2006). It demonstrated pharmacodynamic differences among the three examined strains despite the same MICs while the *in vitro* pharmacokinetic parameters were similar to those observed in humans for all the three drugs.

2.3.1.1 Voriconazole

Voriconazole is currently the drug of choice for the treatment of aspergillosis (Mayr and Lass-Florl, 2011). In general, the MICs of voriconazole for most of the isolates of *A. fumigatus*, *A. flavus*, and *A. terreus* are similar (Scheven and Schwegler, 1995). However, there are significant differences in the *in vivo* activities of voriconazole against infections caused by these three *Aspergillus* species (Takemoto et al, 2009; Warn et al, 2006). Although each isolate of the three *Aspergillus* species used in the present study had the same MIC (0.5 mg/liter), the new *in vitro* PK/PD model revealed significant pharmacodynamic differences, with the greatest efficacy of voriconazole found against *A. fumigatus* and less against *A. terreus*; whereas no activity against *A. flavus* was detected.

Voriconazole at a dosage of 10 mg/kg, which results in a similar drug exposure in mice as standard doses in humans, is active against *A. fumigatus* (Takemoto et al 2009, Murphy et al 1997) but not against *A. flavus* (Warn et al 2006) infections in murine models of invasive aspergillosis. In clinical studies, the survival rate of patients with invasive aspergillosis by *A. terreus* is generally lower than in infections with *A. fumigatus* after treatment with voriconazole (Herbrecht et al 2002, Steinbach et al 2004). Of note, voriconazole showed better activity than other antifungals against *A. terreus* (Steinbach et al 2004) as in our study. These in vivo and clinical data suggest that voriconazole may be more active against *A. fumigatus* than against *A. terreus* and may have little activity against *A. flavus*, in agreement with the findings of the new in vitro model. Thus, the new in vitro pharmacokinetic/pharmacodynamic model revealed clinical and in vivo relevant pharmacodynamic differences of voriconazole against the three *Aspergillus* spp.

The voriconazole $fAUC_{0-24}$ (AUC based on free non-protein bound drug) associated with near maximum activity differed among the three *Aspergillus* spp. with $fAUC_{0-24}$ of 18.9 (14.4-23.1) mg.h/l for *A. fumigatus*, 26.6 (21.1-32.9) mg.h/l for *A. flavus* and 36.2 (27.8-45.7) mg.h/l for *A. terreus*. Bridging these data with human PK showed that the standard dosage of 4 mg/kg was associated with a high median % target attainment for *A. fumigatus* (80%) but not for *A. flavus* (63%) and *A. terreus* (36%), although a wide range of % target attainment was observed reflecting the large inter-patient variation of voriconazole $fAUC_{0-12}$.

In vitro PKPD analysis may reveal differences in antifungal activity that cannot be predicted by an MIC value. Studying the effect of decreasing concentrations of voriconazole provides information about drug pharmacodynamic properties related with sub-MIC effect, post-antifungal effect, time- and concentration-dependent activities. These effects can be quantified by a surrogate marker of fungal growth based on galactomannan production kinetics which captures any difference on the above antifungal effects. The growth rate may be an important determinant of antifungal activity. Voriconazole activity was found to be correlated with growth rates of *Candida* isolates as determined in a pharmacokinetic model (Li et al, 2008) and of *Aspergillus* species using a microdilution assay measuring metabolic activity (Antachopoulos et al, 2007). The three *Aspergillus* species are characterized by different growth rates with *A. terreus* growing slower and *A.*

flavus faster than *A. fumigatus* (Meletiadis et al, 2003; Walsh et al, 2003). Indeed, in the present model a larger voriconazole $fAUC_{0-24}$ was required to inhibit the slow growing *A. terreus* than the other two species. However, the $fAUC_{0-24}$ against the slower growing *A. fumigatus* was smaller than the $fAUC_{0-24}$ against the faster growing *A. flavus* indicating that other factors influence voriconazole activity in the present model such as time dependent effects.

Time-dependent activity of voriconazole inhibition may differ for each *Aspergillus* species. Exposure of *Aspergillus* conidia to concentrations near voriconazole MIC for 6 h resulted in significant amount of fungal growth for *A. terreus* (64%), *A. flavus* (24%) and *A. fumigatus* (15%) isolates (Antachopoulos et al, 2007). After 8h of exposure, voriconazole MICs against *A. fumigatus* isolates were lower than the respective MICs after 48h of incubation whereas the MICs against *A. flavus* isolates remained the same, indicating that voriconazole needs more time to act against *A. flavus*. This may explain the smaller $fAUC_{0-24}$ observed in the present model for *A. fumigatus* in comparison to *A. flavus*.

In agreement with a differential voriconazole time-dependent inhibition among species is the fact that complete inhibition of galactomannan production was not observed for any of the strains tested in the present dynamic model. This effect may be related to drug mechanism of action since azole-induced complete inhibition of ergosterol synthesis requires at least 1 h and complete exchange of ergosterol by its methylated precursors occurs after about 6 h of azole exposure (Scheven et al, 1995). The kinetics of voriconazole inhibition may differ among *Aspergillus* species as reflected in the present dynamic model by the different maxima of galactomannan production for each species. These differences were taken into account during the analysis by normalization of data.

In order to validate the present in vitro PKPD model, the obtained results were compared with those found in a murine model of experimental aspergillosis (Mavridou et al, 2010). In the latter, voriconazole efficacy was tested against four *A. fumigatus* isolates demonstrating increasing MIC values. The AUC/MIC associated with 90% survival in mice was 36.4, which was very close to the AUC/MIC of 37.8 associated with near maximum activity (i.e. 10% fungal growth) in the present study. Voriconazole dosage of 10 and 40 mg/kg, which corresponded to an $fAUC_{0-24}$ of 1 and 20 mg.h/ml, against a wild-type *A. fumigatus* isolate resulted in 90% and 0% mortality, respectively (Mavridou et al, 2010).

Similarly in our in vitro dynamic model, $fAUC_{0-24}$ of 1 and 20 mg.h/ml (0 and 1.3 $\log_{10}$ $fAUC_{0-24}$, respectively) corresponded to 100% and 10% of fungal growth. Finally, voriconazole at a dosage of 10 mg/kg was 296 active against *A. fumigatus* (Murphy et al, 1997; Takemoto et al, 2009) but not *A. flavus* (Warn et al, 2006) infections in murine models of invasive aspergillosis. Likewise, the present study demonstrated that voriconazole was more active against *A. fumigatus* than *A. flavus*. Thus, the results of the in vitro dynamic model can predict the outcome of voriconazole treatment in vivo.

The results of the PKPD model are in agreement with clinical data since the % target attainment (32-97%) for the 4 mg/kg dosage of voriconazole was similar to the survival rates among patients with *A. fumigatus* infections who were treated with standard dosing (50-80%) (Baddley et al, 2009; Herbrecht et al, 2002). In addition, the median % target attainment for this species (80%) was very close to the median 6-week survival (~75%) previously observed in hematological patients infected by *A. fumigatus* isolates with MICs corresponding to 0.5 mg/l (Baddley et al, 2009). The AUC/MIC index of 37.8 which found to be associated with near maximum activity for *A. fumigatus* in the present study can be reached with standard dosing of 4 mg/kg for isolates with MIC 0.5 mg/l supporting thus the voriconazole ECVs of 1 mg/l (Espinel-Ingroff et al, 2010). Regarding the other species, the survival rate of patients with invasive aspergilllosis due to *A. terreus* was generally lower than due to *A. fumigatus* after treatment with voriconazole (Herbrecht et al, 2002; Steinbach et al, 2004). This was also reflected in the present study by a higher % target attainment for *A. fumigatus* compared to *A. terreus*. These correlations provide further clinical validation of the in vitro model.

2.3.1.2 Amphotericin B

Amphotericin B was for decades the treatment of choice for aspergillosis. Clinical and experimental data indicated differences in efficacy against infections caused by various *Aspergillus* species (Denning et al. 1998). Lack of clinical efficacy, however, was not associated with significantly increased MICs (Johnson et al. 2000; Lionakis et al, 2005; Mosquera et al. 2001), which are similar for all three species, with slightly higher MICs of amphotericin B for *A. terreus* (Meletiadis et al. 2007). In the present study, the *Aspergillus* isolates exhibited the same MIC (1 mg/liter), classified as susceptible (Cantón et al, 2009),

suggesting similar efficacies of amphotericin B. However, the new *in vitro* model revealed differences in the efficacy of amphotericin B against the three *Aspergillus* species, with the following order: *A. fumigatus* > *A. flavus* > *A. terreus*. These findings are in agreement with previous comparative animal studies where treatment with amphotericin B was more effective against infection cause by *A. fumigatus* than against infection with *A. flavus* and less effective against infection with *A. terreus* (Odds et al, 1998; Walsh et al, 2003).

In vitro simulation of amphotericin B pharmacokinetics is a challenge because of the inherent characteristics of the drug to degrade after incubation at 35^{o}C and to attach to surfaces (Lewis et al, 2006). In order to minimize these two effects, the in vitro pkpd model was covered with aluminum foils to avoid light exposure and the plastic surfaces were kept at minimum (only the tubing) since beakers were from glass and the dialysis tube from cellulose. In order to ensure that amphotericin B was not attached to dialysis tubes or any other component of the model and was not degraded during the experiments, preliminary experiments were performed measuring amphotericin B concentrations after incubation inside dialysis tubes and showed that the loss was minimal (data not shown). However, when fungi were added inside the dialysis tubes, a rapid followed by a slower reduction of amphotericin B concentration was observed resulting in the biphasic time-concentration profile approximating the one observed in humans.

The findings of the present study are in agreement with previous time-kill assays where supra-MIC (4x and 20xMIC) concentrations of amphotericin B killed *A. fumigatus* but not *A. terreus* (Walsh et al, 2003). Minimal fungicidal concentrations of amphotericin B were similar against *A. fumigatus* and *A. flavus* and much higher against *A. terreus* while MFC/MIC ratios of *A. fumigatus* were lower than against *A. flavus* (Meletiadis et al, 2007). In addition, the three species ware previously found to differ also in the post-drug exposure effects since 4x and 1xMIC of amphotericin B demonstrated >4h post antifungal effect against *A. fumigatus* and <4h against *A. flavus* and *A. terreus* (Vitale et al, 2002). Time-dependent activity of amphotericin B inhibition was also differed among the three *Aspergillus* species (Antachopoulos et al, 2007). Exposure of *Aspergillus* conidia to supra-MIC concentrations for 8h resulted in significant amount of metabolic activity for *A. terreus* (16%), less for *A. flavus* (8%) and lesser for *A. fumigatus* (5%) isolates. Furthermore, despite the same concentration-effect curves of amphotericin B for *A. fumigatus* and *A.*

flavus at 48h, the concentration-effect curve after only 8h of exposure to amphotericin B were shifted to the left for *A. fumigatus* while remained unchanged for *A. flavus* indicating that amphotericin B inhibitory activity is faster against *A. fumigatus* than *A. flavus* species (Antachopoulos et al, 2007).

The order of the in vitro activity of amphotericin B found with the present model *A. fumigatus>A. flavus>A. terreus* could be explained by the fast inhibitory action and increased killing rate against *A. fumigatus*, the slower inhibitory action and less killing against *A. flavus* and the slowest inhibitory action and no killing against *A. terreus*. In particular, the delay of *A. fumigatus* but not of *A. flavus* galactomannan production at C_{max}=0.6 mg/l indicate a strong sub-MIC effect of amphotericin B against the former species. Although there are no data on sub-MIC effects of amphotericin B against *Aspergillus* spp. such effects were described against *Candida* spp. (Garcia et al, 2002). At C_{max}=1.2 mg/l, galactomannan was detected after 48h incubation for *A. fumigatus* reflecting the minimal fungicidal action at this concentration (usually observed at 2xMIC) (Meletiadis et al, 2007) and the long post-antifungal effect observed at 1xMC (Vitale et al, 2002) together with the sub-MIC effect after amphotericin B concentrations fell below the MIC. The absence of galactomannan production at concentrations >2xMIC reflects the fungicidal activity amphotericin B demonstrated at time-kill assays (Walsh et al, 2003). For *A. flavus*, galactomannan was detected at C_{max}=4.8, 2.4 and 1.2 mg/l after 48h, 24h and 6h as soon as the concentration fell below MIC reflecting the absence of killing and post-antifungal effects as previously described (Vitale et al, 2002). Of note, at all three doses galactomannan production was detected after 4h despite amphotericin B concentrations being higher than the MIC reflecting the slow inhibitory action of amphotericin B against this species, as previously was found (Antachopoulos et al, 2007). Finally, the modest delay in galactomannan production of *A. terreus* at all doses may reflect the lack of killing, post-antifungal and sub-MIC effects and the slow inhibitory action against this species. Thus, single-dose pharmacodynamics in the present in vitro pkpd model where amphotericin B concentrations decrease over time may reflect concentration- and time-dependent inhibitory and killing activities described by MFC, time-kill and post-antifungal effect assays.

Lack of clinical efficacy, however, was not associated with significantly increased amphotericin B MICs values (Johnson et al, 2000; Lionakis et al, 2005, Mosquera et al, 2001), which were similar for all three species (Baddley et al, 2009; Meletiadis et al, 2007). The only animal studies demonstrated that treatment with amphotericin B was more effective against infection caused by *A. fumigatus* than infection with *A. flavus* and less effective against infection with *A. terreus* (Odds et al, 1998; Walsh et al, 2003). Clinical studies also demonstrated a higher mortality rate of infections by *A. terreus* compared to those by *A. fumigatus* despite amphotericin B therapy (Lass-Florl et al, 1998; Steinbach et al, 2004). It seems that the new in vitro model, described here, may better characterize the pharmacodynamic characteristics of amphotericin B for the major *Aspergillus* species tested than conventional in vitro susceptibility systems.

2.3.1.3 Caspofungin

Caspofungin is used as salvage therapy against invasive aspergillosis, although recent studies suggest its effectiveness as firstline therapy (Herbrecht et al, 2010). In the new *in vitro* model, caspofungin did not demonstrate any effect on the growth of an *Aspergillus* species other than *A. terreus*, for which galactomannan production was simply delayed for 2.83 h. Caspofungin inhibits 1,3-β-D-glucan synthesis, which results in distortion of the cell wall and suppression of growth. Galactomannan may be released during the process of cell wall disruption. Consequently, galactomannan levels may remain high despite effective antifungal activity. Thus, for the pharmacodynamic study of the actions of caspofungin and other echinocandins, measurement of galactomannan production appears not to be informative neither *in vitro* nor *in vivo* (Petraitiene et al, 2002). Another biomarker is needed to monitor the pharmacodynamics of echinocandins. An alternative endpoint for determining the activity of antifungal drugs, and particularly of echinocandins, may be PCR CEs.

2.3.1.4. Voriconazole and Amphotericin combination

The combination of voriconazole with amphotericin B exerted antagonistic effects (-4 to -13%) in the wider range of concentrations tested (VOR 1.7 mg/L with AMB 0.6, 0.3 and

0.1 mg/L), whereas some synergistic interactions at a percentage of 26% were observed at amphotericin B and voriconazole doses with Cmax 0.1 and 1.7 mg/L, respectively. These concentrations correspond to the free drug levels of amphotericin B and voriconazole achieved in human plasma after invravenous administration of the standard doses 1 mg/kg and 4 mg/kg.

2.3.2. CONCLUSION

In conclusion, in the present study, we developed and for the first time reported an *in vitro* PK/PD model which simulates the pharmacokinetics of three major antifungal drugs in humans and assesses their efficacy against three clinically significant *Aspergillus* species. Because total drug concentrations were simulated in the *in vitro* PK/PD model, the drug that is available for microbiologic activity may be overestimated due to protein binding *in vivo*. Furthermore, this model should be validated with results of *in vivo* antifungal efficacy from animal experiments. This model can be adapted to incorporate serum, neutrophils, and other host factors that may affect antifungal pharmacodynamics.

In the in vitro PK-PD modeling of voriconazole, higher voriconazole exposure was required to inhibit *A. terreus* than *A. flavus* and *A. fumigatus*. Based on the *in vitro* exposure-effect relationships found in the novel PK/PD model, a standard dosage of voriconazole may be adequate for most patients with *A. fumigatus* infections; higher drug exposure appears to be required for *A. flavus* and *A. terreus* infections. This could be achieved using a higher voriconazole dosage or by therapeutic drug monitoring in patients receiving the standard dosage given the wide distribution of voriconazole $fAUC_{0-12}$ among them. Optimizing voriconazole exposure in order to obtain $fAUC_{0-12}$ at the upper 95% confidence interval limit, would result in % target attainment >83% for all three *Aspergillus* species. Therapeutic drug monitoring for voriconazole is becoming an important tool to improve efficacy and safety of this agent (Bruggemann et al, 2008). This is corroborated by clinical studies where adequate voriconazole levels have been associated with efficacy, while drug underexposure was associated with worse outcome (Murphy et al, 1997; Pascual et al, 2008). There is a multitude of factors that can influence patient outcomes. In that respect, in vitro PKPD modeling assists in providing information on critical parameters such as drug kinetics and dynamics, thus offering a

powerful tool for estimating the impact of drug underexposure and improving efficacy through drug exposure optimization.

Also, this model simulated well amphotericin B human pharmacokinetics and demonstrated a differential *in vitro* activity against the three *Aspergillus* species that are not reflected by the MICs. These effects may be the sum of concentration- and time-dependent inhibitory/killing activities of amphotericin B which exhibited the greatest activity against *A. fumigatus* and lowest against *A. terreus*. Future studies should focus on testing large collections of isolates in order to describe the distribution of this new pharmacodynamic parameter and optimize it in order to obtain clinically relevant drug exposures. A composite parameter that describes many and different pharmacodynamic effects of antifungal drugs may be more sensitive and representative of antifungal activity than conventional pharmacodynamic parameters.

Caspofungin did not demonstrate any effect on the growth of an *Aspergillus* species other than *A. terreus*, in which galactomannan production was simply delayed. Consequently, galactomannan levels may remain high despite effective antifungal activity. Thus, for pharmacodynamic study of the actions of caspofungin and other echinocandins, measurement of galactomannan production appears not to be informative and another biomarker is needed to monitor the pharmacodynamics of echinocandins.

Moreover, in drug combination experiments, we showed that voriconazole interacted in a concentration-dependent manner with amphotericin B. The combination of voriconazole with a low dose of amphotericin B could enhance overall efficacy or possibly enable effectiveness in the treatment directed against *A. fumigatus* isolates.

In vitro PKPD modeling may assist in providing information on critical parameters such as drug kinetics and dynamics, thus offering a powerful tool for estimating the impact of drug underexposure and improving efficacy through drug exposure optimization. Furthermore, this model seems to describe the in vitro activity of antifungal drugs more reliably than the conventional susceptibility testing methods. The effects observed in the in vitro PK/PD model may be the sum of concentration- and time-dependent inhibitory/killing activities exerted by antifungal drugs. This model can be used to explore more effective antifungal dosing regimens and improve treatment and prognosis of *Aspergillus* infectious.

Future studies should focus on testing large collections of isolates in order to describe the distribution of this new pharmacodynamic parameter and optimize it in order to obtain clinically relevant drug exposures.

2.4.1. SUMMARY

INTRODUCTION

The in vitro activities of antifungal agents against *Aspergillus* spp. are determined by standardized broth microdilution assays where the drug concentration remains constant over time (Cantón et al, 2009). However, fungi are exposed in vivo to fluctuating drug concentrations over time because of absorption, elimination, excretion, metabolism, and distribution processes (Groll et al, 1998). The purposes of this study were to develop an in vitro model that simulates the pharmacokinetics of amphotericin B, voriconazole, and caspofungin in humans after the intravenous administration of standard doses and to study the effects of standard dosing regimes and explore alternative single and combination dosing regimens against clinical isolates of *A. fumigatus, A. flavus,* and *A. terreus*.

MATERIALS AND METHODS

Three isolates of *A. fumigatus*, *A. flavus*, and *A. terreus* obtained from patients with invasive pulmonary aspergillosis with amphotericin B and voriconazole CLSI MICs 1 mg/l and caspofungin MECs 0.5 mg/l. The *in vitro* PKPD simulation model consisted of a 10-ml-volume dialysis tube (internal compartment IC) made of a semipermeable cellulose membrane (Float-A-Lyzer; Spectrum Europe B.V., Breda, The Netherlands) allowing the free diffusion of small molecules (MW=20 kDa). This is placed in a glass beaker containing 700 ml medium (external compartment EC) the content of which is continuously diluted by a peristaltic pump (Minipuls Evolution; Gilson Inc., Villiers le Bel, France) removing drug-containing medium from the EC and adding drug-free medium to the EC at a rate equivalent to drug clearance in human plasma. At time zero, the IC was inoculated with 10 ml medium containing $1x10^5$ CFU/ml of *Aspergillus* conidia. The following doses of antifungal agents were simulated in the *in vitro* model: 0.25, 0.5, 1, 2 mg/kg for amphotericin B, 0.5, 1, 1.5 mg/kg for caspofungin, and 3, 4, 5 mg/kg for voriconazole. The GIs in the IC were measured at regular time intervals up to 72 h. Finally, twelve different combinations of amphotericin B and voriconazole were investigated against *A. fumigatus*. The IC was inoculated with a conidial suspension (10^3 CFU/mL), while drug

combinations were injected in both compartments (VOR/12h, AMB/24h). The PD analysis for the interactions studied was described using the Bliss independence model.

RESULTS

Pharmacokinetic analysis. A) Voriconazole. The one compartment pharmacokinetic model described well the drug levels in the IC (R^2>0.97). Intra-and inter-experimental variation of the in vitro PK data was <10 %. In vitro voriconazole pharmacokinetics were close to the target values observed in human plasma after administration of 3, 4 and 5 mg/kg voriconazole with $t_{1/2}$ 5.7-6.5h. **b) Amphotericin B.** The C_{max} and AUCs in the IC for the simulated amphotericin B doses 0.25, 0.5, 1 and 1.5 mg/kg were close ot those observed in humans respectively with $t_{1/2}$,α 0.2-2h, $t_{1/2}$,β 10-17h and $t_{1/2}$,γ 71h. **c) Caspofungin.** In vitro caspofungin pharmacokinetics was closed to the target values observed in human plasma after administration of 0.5, 1 and 2 mg/kg caspofungin with $t_{1/2}$ of 14.8-11.6h as in patients' plasma.

Pharmacodynamic analysis. a) Voriconazole. All three voriconazole doses delayed galactomannan production by *A. fumigatus* whereas only the two highest doses decreased the maximum galactomannan production compared to growth control. In the case of *A. flavus*, there was no delay in galactomannan production, whereas maximum galactomannan production was reduced as voriconazole dose increased. Regarding *A. terreus*, voriconazole delayed galactomannan production whereas maximum galactomannan production was reduced as voriconazole dose increased. Based on the normalized PKPD relationship, the $fAUC_{0-24}$ associated with near-maximum activity (10% AUC_{GI}) was 18.9 (14.4-23.1) mg.h/l against *A. fumigatus*, 26.6 (21.1-32.9) mg.h/l against *A. flavus* and 36.2 (27.8-45.7) mg.h/l against *A. terreus* ($F_{2,19}$=17.22, p<0.0001). These associated with 90% survival in an animal model of experimental aspergillosis (Mavridou, et al, 2010); thus providing a validation of the in vitro PKPD model. Bridging the in vitro PKPD data with human PK data, low median % target attainment (<45%) was found for 3 mg/kg voriconazole dosing for all species. The standard dosage of 4 mg/kg was associated with high median % target attainment (80%) for *A. fumigatus* but not for *A. flavus* and *A. terreus* for which as low as 17% and 6% target attainment, respectively, was found for $fAUC_{0-12}$ at the lower 95% confidence interval limit observed in patients. **b) Amphotericin B.** The GI–time curves were characterized by the same maximum but

different rates of galactomannan production for the different amphotericin B doses and *Aspergillus* species. Among all species and doses tested, complete inhibition of galactomannan production was observed only against *A. fumigatus* with amphotericin B doses corresponding to C_{max} ≥2.4 mg/l whereas at lower doses, a significant delay in galactomannan production was observed. For *A. flavus*, there was no complete inhibition but a progressive delay of galactomannan production with increasing amphotericin B doses. For *A. terreus*, the delay in galactomannan production was modest. **c) Caspofungin.** Caspofungin did not have any effect on galactomannan production whereas when *Aspergillus* DNA was measured by real-time PCR, 2.2-, 0.9-, and 1.6-log reductions in the CEs of *A. fumigatus*, *A. flavus*, and *A. terreus*, respectively, were observed after 6 h of incubation in the *in vitro* PK/PD model with (C_{max}=10 mg/L) and extend to 8h with (C_{max}=20 mg/L) in the presence of caspofungin compared to those of the drug-free controls.

Combination therapy. The combination of voriconazole with amphotericin B exerted antagonistic effects (-4 to -13%) in a wide range of doses tested with C_{max} 1.7, 3.4 and 7.8 mg/L for voriconazole and 0.6, 0.3 and 0.1 mg/L for amphotericin B, whereas some synergistic interactions at a percentage of 26% were observed at lower amphotericin B and voriconazole doses with Cmax 0.1 and 1.7 mg/L, respectively. These concentrations correspond to the free drug levels of amphotericin B and voriconazole achieved in human plasma after invravenous administration of the standard doses 1 mg/kg and 4 mg/kg.

DISCUSSION

Here we report for the first time, to our knowledge, a new *in vitro* pharmacokinetic model for *Aspergillus* species that simulates the human plasma pharmacokinetics of three major anti-*Aspergillus* agents (voriconazole, amphotericin B and caspofungin). For the pharmacodynamic analysis of these regimens, galactomannan production was determined as a surrogate marker of fungal growth. The three *Aspergillus* strains tested in this model had the same MICs and MECs and represent the most clinically significant species (*A. fumigatus*, *A. flavus*, and *A. terreus*). The new in vitro model revealed pharmacodynamics difference among the three species that were not apparent with the conventional in vitro susceptibility testing broth microdilution methodologies where the drug concentration of antifungal agents remained constant over time (Lewis et al, 2006).

Although each isolate of the three *Aspergillus* species used in the present study had the same MIC (0.5 mg/liter), the new *in vitro* PK/PD model revealed significant pharmacodynamic differences, with the greatest efficacy of voriconazole found against *A. fumigatus* and less against *A. terreus*; whereas no activity against *A. flavus* was detected. Furthermore, the voriconazole $fAUC_{0-24}$/MIC associated with near maximum activity was 18.9 (14.4-23.1) mg.h/l for *A. fumigatus*, which is very close of the PKPD target found in in vivo experiments withy mice (Mavridou et al 2005) validating the results of the in vitro model. Bridging these PKPD data with human PK showed that the standard dosage of 4 mg/kg was associated with a high median % target attainment for *A. fumigatus* (80%) but not for *A. flavus* (63%) and *A. terreus* (36%), although a wide range of % target attainment was observed reflecting the large inter-patient variation of voriconazole $fAUC_{0-12}$. Therapeutic drug monitoring or use of higher dosages may increase the efficacy of voriconazole therapy for the non-fumigatus *Aspergillus* species.

In the present study, the *Aspergillus* isolates exhibited the same Amphotericin B MIC (1 mg/liter), classified as susceptible (Cantón et al, 2009), suggesting similar efficacies of amphotericin B. However, the new *in vitro* model revealed differences in the efficacy of amphotericin B against the three *Aspergillus* species, with the following order: *A. fumigatus* > *A. flavus* > *A. terreus*. These findings are in agreement with previous comparative animal studies where treatment with amphotericin B was more effective against infection cause by *A. fumigatus* than against infection with *A. flavus* and less effective against infection with *A. terreus* (Odds et al, 1998; Walsh et al, 2003). The new in vitro model may capture different pharmacodynamic effects beyond inhibitory actions that are described by the MICs such as fungicidal, time-dependenent and post-antifungal effects.

In the new *in vitro* model, caspofungin did not demonstrate any effect on the galactomannan production by *Aspergillus* species other than *A. terreus*. Galactomannan may be released during the process of cell wall disruption. Consequently, galactomannan levels may remain high despite effective antifungal activity. Thus, for pharmacodynamic study of the actions of caspofungin and other echinocandins, measurement of galactomannan production appears not to be informative either *in vitro* and *in vivo* (Petraitiene et al, 2002). Another biomarker is needed to monitor the pharmacodynamics

of echinocandins. An alternative endpoint for determining the activity of antifungal drugs, and particularly of echinocandins, may be PCR CEs.

CONCUSION

In conclusion, in the present study, we developed and for the first time reported an *in vitro* PK/PD model which simulates the pharmacokinetics of three major antifungal drugs in humans and assesses their efficacy against three clinically significant *Aspergillus* species. In vitro PK-PD modeling voriconazole showed that higher voriconazole exposure may be required in order to inhibit *A. terreus* than *A. flavus* and *A. fumigatus*. Also, this model simulated well amphotericin B human pharmacokinetics and demonstrated a differential in vitro activity against the three *Aspergillus* species that are not reflected by the MICs. These effects may be the sum of concentration- and time-dependent inhibitory/killing activities of amphotericin B which exhibited the greatest activity against *A. fumigatus* and lowest against *A. terreus*. About caspofungin, it did not demonstrate any effect on the growth of an *Aspergillus* species other than *A. terreus*, in which galactomannan production was simply delayed. Consequently, galactomannan levels may remain high despite effective antifungal activity. Thus, another biomarker is needed to monitor the pharmacodynamics of echinocandins.

Moreover, the model was applied to simulate the pharmacokinetics of antifungal combination therapy with voriconazole and amphotericin B which is commonly used to treat refractory aspergillosis. The model showed that voriconazole interacted in a concentration-dependent manner with amphotericin B with synergistic interactions observed at concentrations that corresponded to the free drugs levels obtained after standard dosing regimes. Thus, the voriconazole+amphotericin B combination may enhance overall antifungal efficacy while therapeutic drug monitoring of combination regimes may increase synergistic effects against *A. fumigatus* isolates.

2.4.2. ΠΕΡΙΛΗΨΗ

ΕΙΣΑΓΩΓΗ

Η in vitro δραστικότητα των αντιμυκητιακών έναντι ασπεργίλλων προσδιορίζεται με προτυποποιημένες μεθόδους στις οποίες η συγκέντρωση του φαρμάκου παραμένει σταθερή. Ωστόσο, η ενδοφλέβια χορήγηση αντιμυκητιασικών οδηγεί σε συγκεντρώσεις στο πλάσμα του αίματος που ελαττώνονται λόγω της απέκκρισης, του μεταβολισμού και της κατανομής του φαρμάκου. Επομένως, in vivo ο μύκητας εκτίθεται σε μη σταθερές συγκεντρώσεις φαρμάκων, γεγονός που δεν αναπαράγεται με τις κλασσικές μεθόδους προσδιορισμού των MIC. Σκοποί της παρουσας διατριβής ήταν ανάπτυξη ενός συστήματος προσομοίωσης της φαρμακοκινητικής της αμφοτερικίνης Β, της βορικοναζόλης και της κασποφουγκίνης όπως αυτή παρατηρείται μετά από ενδοφλέβια χορήγηση της ενδεδειγμένης δοσολογίας στον άνθρωπο, καθώς και η μελέτη της δράσης των διαφόρων δοσολογικών σχημάτων αυτών μόνα ή σε συνδυασμό έναντι κλινικών στελεχών *A. fumigatus*, *A. flavus* και *A. terreus*.

ΥΛΙΚΑ ΚΑΙ ΜΕΘΟΔΟΙ

Χρησιμοποιήθηκαν τρία κλινικά στελέχη *A. fumigatus*, *A. flavus* και *A. terreus* που απομονώθηκαν από ασθενείς με διηθητική πνευμονική ασπεργίλλωση με MICs 1 mg/l για την αμφοτερικίνη Β και 0.5 mg/l για την βορικοναζόλη, και MECs για την κασποφουγκίνη 0.5 mg/l**.** Το in vitro φαρμακοκινητική/φαρμακοδυναμική μοντέλο προσομοίωσης αποτελείται από ένα σωλήνα διαπίδυσης 10-ml όγκου (εσωτερικό διαμέρισμα ΕΣΔ) κατασκευασμένο από μια ημιπερατή μεμβράνη κυτταρίνης (Float-A-Lyzer; Breda, The Netherlands) επιτρέποντας την ελεύθερη διάχυση μικρών μορίων (μοριακού βάρους, <20 kDa). Αυτό τοποθετείται σε ένα γυάλινο ποτήρι που περιέχει 700 ml θρεπτικού μέσου (εξωτερικό διαμέρισμα ΕΞΔ) το περιεχόμενο του οποίου αραιώνεται συνεχώς από μία περισταλτική αντλία (Minipuls Evolution; Gilson Inc, Villiers le Bel, France) απομακρύνοντας θρεπτικό υλικό που περιέχει το φάρμακο μέσα από το ΕΞΔ και προσθέτοντας θρεπτικό υλικό χωρίς φάρμακο μέσο στο ΕΞΔ με ρυθμό ίσο με τον ρυθμό κάθαρσης του φαρμάκου στο ανθρώπινο πλάσμα. Κατά το χρόνο μηδέν, το ΕΣΔ εμβολιάστηκε με 10 ml θρεπτικού που περιείχε $1X10^5$ CFU/ml του μύκητα. Το φάρμακο ενίεται στο ΕΞΔ και στο ΕΣΔ ενώ η αντλία αραιώνει τη συγκέντρωση του φαρμάκου στο

ΕΞΔ ώστε ο μέσος χρόνος ημίσειας ζωής να αντιστοιχεί με αυτόν που παρατηρείται στο ανθρώπινο πλάσμα μετά την ενδοφλέβια χορήγηση αμφοτερικίνης Β, κασποφουγκίνης και βορικοναζόλης σύμφωνα με προηγούμενες κλινικές μελέτες. Το ΕΞΔ τοποθετείται σε θερμαινόμενο μαγνητικό αναδευτήρα (37^{o}C). Πριν την έναρξη και κατά την διάρκεια κάθε πειράματος, ελέγχεται η θερμοκρασία και ο ρυθμός ροής. Προσομοιώθηκαν οι παρακάτω δοσολογίες αντιμυκητιασικών φαρμάκων 0.25, 0.5, 1, 2 mg/kg για την αμφοτερικίνη Β, 0.5, 1, 1.5 mg/kg για την κασποφουγκίνη, και 2, 3, 4, 5 mg/kg για τη βορικοναζόλη. Τα επίπεδα γαλακτομαννάνης μετρήθηκαν με ανοσοενζυμική μέθοδο sandwich ELISA (Platelia Aspergillus, Bio-Rad Laboratories, Αθήνα, Ελλάδα), και τα αποτελέσματα εκφράστηκαν ως δείκτης γαλακτομαννάνης (GI) σύμφωνα με τις οδηγίες του κατασκευαστή. Ο GI μετρήθηκε σε ΕΣΔ που εμβολιάσθηκαν με 10^5 CFU/ml *A. fumigatus*, *A. flavus*, ή *A. terreus* σε τακτά χρονικά διαστήματα μέχρι τις 72 ώρες. Τα φαρμακοδυναμικά δεδομένα υποβλήθηκαν σε ανάλυση μη γραμμικής παλινδρόμησης με βάση το σιγμοειδές Emax μοντέλο. Διαφορετικές δοσολογίες συνδυαστικής θεραπείας με βορικοναζόλη και αμφοτερικίνη Β προσομοιώθηκαν στο νέο in vitro PKPD μοντέλο με χρόνους ημιζωής 6 ώρες για την βορικοναζόλη και 12 ώρες για την β φάση της αμφοτερικίνης Β. Το ΕΣΔ εμβολιάστηκε με κονιδιακό εναιώρημα (10^3 CFU/mL), ενώ τα διαλύματα φαρμάκου βορικοναζόλης και αμφοτερικίνης Β εγχύθηκαν στα δύο διαμερίσματα δις και άπαξ ημερησίως, αντίστοιχα. Οι φαρμακοδυναμικές αλληλεπιδράσεις αναλύθηκαν με το μοντέλο ανεξαρτησίας κατά Bliss.

ΑΠΟΤΕΛΕΣΜΑΤΑ

Φαρμακοκινητική ανάλυση. α) Βορικοναζόλη. Το μονο-διαμερισματικό φαρμακοκινητικού μοντέλου περιέγραφε καλά τα επίπεδα του φαρμάκου στο ΕΣΔ (R^2> 0.97). Η διακύμανση των in vitro PK δεδομένων μεταξύ των πειραμάτων ήταν <10%. Η in vitro φαρμακοκινητική της βορικοναζόλης ήταν κοντά με τις τιμές στόχους που παρατηρήθηκαν στο ανθρώπινο πλάσμα μετά από χορήγηση των 3, 4 και 5 mg/kg βορικοναζόλης. Ο χρόνος ημιζωής $t_{1/2}$ της βορικοναζόλης στο in vitro μοντέλο ήταν 5.7-6,5 ώρες.. **β) Αμφοτερικίνη Β**. Η C_{max} και η AUCs στο ΕΣΔ των προσομοιωμένων δοσολογιών 0.25, 0.5, 1 και 1.5 mg/kg ήταν πολύ κοντά στις αντίστοιχες τιμές στον άνθρωπινο πλάσμα με $t_{1/2,\alpha}$ 0.2-2h, $t_{1/2,\beta}$ 10-17h και ο $t_{1/2,\gamma}$ 71h για την αμφοτερικίνη Β,

αντιστοίχως. **γ) Κασποφουγκίνη**. Η in vitro φαρμακοκινητική της κασποφουγκίνης προσέγγισε τις τιμές στόχους που παρατηρήθηκαν στο ανθρώπινο πλάσμα μετά από χορήγηση 0,5, 1 και 2 mg/kg caspofungin με χρόνο ημιζωής 11.6-14.8h.

Φαρμακοδυναμική ανάλυση. α) Βορικοναζόλη. Όλες οι τρεις προσομοιωμένες δόσεις βορικοναζόλης καθυστέρησαν την παραγωγή γαλακτομαννάνης από τον *A. fugimatus* ενώ μόνο οι δύο υψηλότερες δόσεις μείωσαν την μέγιστη παραγωγή γαλακτομαννάνης σε σύγκριση με τον μάρτυρα. Στην περίπτωση του *A. flavus*, η βορικοναζόλη δεν επηρέασε τον ρυθμό παραγωγής της γαλακτομαννάνης, ενώ η μέγιστη παραγωγή γαλακτομαννάνης μειώθηκε συναρτήσει της δόσης. Όσον αφορά τον *A. terreus*, η βορικοναζόλη καθυστέρησε την παραγωγή γαλακτομαννάνης και μείωσε την μέγιστη παραγωγή. Με βάση την κανονικοποιημένη PKPD σχέση, η $fAUC_{0-24}$ που σχετίζεται με τη μέγιστη δραστικότητα (10% ανάπτυξη) ήταν 18.9 (14.4–23.1) mg.h/l για *A. fumigatus*, 26.6 (21.1 – 32.9) mg.h/l για *A. flavus* και 36.2 (27.8–45.7) mg.h/l για *A. terreus* ($F_{2, 19}$ = 17.22, p<0,0001). Η τιμή $fAUC_{0-24}$ 18.9 συμφωνεί με την in vivo τιμή $fAUC_{0-24}$ που σχετίζεται με 90% επιβίωση σε ένα ζωικό μοντέλο πειραματικής ασπεργίλλωσης (Mavridou et al 2010) παρέχοντας έτσι μια επικύρωση του in vitro PKPD μοντέλο. Συσχετίζοντας το in vitro PKPD δεδομένα με τα ανθρώπινα PK δεδομένα, το % των ασθενών που πέτυχε το φαρμακοδυναμικό στόχο μέγιστης δραστικότητας με την ενδεδειγμένη δοσολογία των 4 mg/kg ήταν υψηλό για τον *A. fumigatus* (80%) αλλά όχι για τους *A. flavus* και *A. terreus* για τους οποίους το ποσοστό επίτευξης του στόχου αυξήθηκε >68% για την δοσολογία των 5 mg/kg. **β) Αμφοτερισίνης Β**. Οι καμπύλες GI-χρόνου υπό την επίδραση διαφορετικών δόσεων αμφοτερικίνης Β (R^2> 0,86) χαρακτηριζόταν από την ίδια την τιμή E_{max}, αλλά διαφορετικές κλίσεις και T_{50}s για τις διάφορες δόσεις αμφοτερικίνης Β και είδη *Aspergillus*. Μεταξύ όλων των ειδών και των δόσεων, πλήρης αναστολή της παραγωγής γαλακτομαννάνης παρατηρήθηκε μόνο ενάντια σε *A. fumigatus* με δόσεις αμφοτερικίνη Β που αντιστοιχούν σε Cmax ≥ 2,4 mg / l. Σε χαμηλότερες δόσεις, παρατηρήθηκε σημαντική καθυστέρηση στην παραγωγή γαλακτομαννάνης. Για A. flavus, δεν υπήρχε πλήρης αναστολή, αλλά μια προοδευτική καθυστέρηση της παραγωγής γαλακτομαννάνης με αυξανόμενες δόσεις αμφοτερικίνης Β. Για *A. terreus*, η καθυστέρηση στην παραγωγή γαλακτομαννάνης ήταν μέτρια. **γ) Κασποφουγκίνη**. Η κασποφουγκίνη δεν είχε σημνατική επίδραση στην παραγωγή γαλακτομαννάνης ενώ όταν το DNA των Aspergillus

μετρήθηκε με PCR πραγματικού χρόνου, παρατηρήθηκαν 2.2-, 0.9-και 1.6-log μειώσεις στα κονιδιακά ισοδύναμα (CE) των *A. fumigatus*, *A. flavus* και *A. terreus*, αντίστοιχα, μετά από 6 ώρες επώασης στο in vitro PKPD μοντέλο με C_{max}=10 mg/L κασποφουγκίνης σε σύγκριση με τον χωρίς φάρμακο μάρτυρα.

Συνδυστική θεραπεία. Ο συνδυασμός της βορικοναζόλης με αμφοτερικίνη Β ήταν ανταγωνιστικός (-4 έως -13%) στις περισσότερες δοσολογίες που δοκιμάσθηκαν με C_{max} 1.7, 3.4 και 7.2 mg/l βορικοναζόλης και 0,6, 0,3 και 0,1 mg/L αμφοτερικίνης Β, ενώ συνεργικές αλληλεπιδράσεις σε ποσοστό από 26% παρατηρήθηκαν στις χαμηλότερες δοσολογίας αμφοτερικίνης Β και βορικοναζόλης με C_{max} 0.1 mg/L και 1.7 mg/l, αντίστοιχα. Οι τελευταίες συγκεντρώσεις αντιστοιχούν στα ελεύθερα επίπεδα των φαρμάκων που επιτυγχάνονται στο αίμα μετά από ενδοφλέβια χορήγηση των ενδεδειγμένων δοσολογιών.

ΣΥΖΗΤΗΣΗ

Η παρούσα διατριβή περιγρέφει για πρώτη φορά, κατά τη γνώση μας, ένα νέο in vitro φαρμακοκινητικό μοντέλο για είδη *Aspergillus* που μιμείται την ανθρώπινη φαρμακοκινητική στο πλάσμα τριών αντιμυκητιακών φαρμάκων τη βορικοναζόλη, αμφοτερικίνη Β και κασποφουγκίνη μετά από ενδοφλέβια χορήγηση. Για τη φαρμακοδυναμική ανάλυση αυτών των σχημάτων, η παραγωγή γαλακτομαννάνης χρησιμοποιήθηκε ως δείκτης μυκητιακής ανάπτυξης. Τρία στελέχη *Aspergillus* που είχαν την ιδια ελάχιστη ανασταλτική συγκέντρωση (MIC) και αντιπροσωπεύουν τα πιο σημαντικά κλινικά είδη *A. fumigatus*, *A. flavus* και *A. terreus* μελετήθηκαν με αυτό το μοντέλο. Με βάση αυτό το μοντέλο παρατηρήθηκαν φαρμακοδυναμικές διαφορές μεταξύ των ειδών οι οποίες δεν ήταν εμφανείς με τους συμβατικούς ελέγχους ευαισθησίας των μικροαραιωσεων σε υγρό ζωμό όπου η συγκέτρωνση του φαρμάκου παραμένιε σταθερή στη διαρκεια του χρονου (Lewis et al, 2006).

Μολονότι κάθε στέλεχος *Aspergillus* που χρησιμοποιήθηκε στην παρούσα μελέτη είχε την ίδια CLSI MIC (0.5 mg/L), το νέο in vitro PKPD μοντέλο αποκάλυψε σημαντικές φαρμακοδυναμικές διαφορές, με τη βορικοναζόλη να εμφανίζει την μεγαλύτερη δραστικότητα έναντι *A. fumigatus* και λιγότερο έναντι *A. terreus* ενώ καμία η μικρότερη δράση ανιχνεύθηκε έναντι του *A. flavus* επιβεβαιώνοντας προηγούμενες in vivo και κλινικές μελέτες (Takemoto et al 2009, Murphy et al 1997, Warn et al 2006, Herbrecht et al 2002, Steinbach et al 2004). Επίσης, PKPD ανάλυση των in vitro δεδομένων του μοντέλου έδειξε

ότι ο PKPD στόχος που σχετίζεται με την μεγίστη δραστικότητα της βορικοναζόλης είναι 18,9 (14,4 - 23,1) mg.h/l $fAUC_{0-24}$/MIC ο οποίος είναι πολύ κοντά στον αντίστοιχο στόχο που βρέθηκε σε ιn vivo πειράματα σε ποντίκια (Mavridou et al 2005) επικυρώνοντας τα αποτελέσματα του in vitro μοντέλου της παρούσας διατριβής. Συσχετίζοντας αυτό τον PKPD στόχο με PK δεδομένα από ανθρώπους, βρέθηκε ότι η τυπική δόση των 4 mg/kg, σχετίστηκε με υψηλά % επίτευξης του στόχου για είδη *A. fugimatus* (80%), αλλά όχι για *A. flavus* (63%) και *A. terreus* (36%) για τα οποία παρατηρήθηκε ένα ευρύ φάσμα % επίτευξης στόχου που αντανακλά την μεγάλη διακύμανση των επιπέδων της βορικοναζόλης μεταξύ των ασθενών. Παρακολούθηση των επιπέδων η χρήση υψηλότερη δόσης βορικοναζόλης θα μπορούσε να αυξήσει την αποτελεσματικότητας του φαρμάκου για αυτά τα είδη.

Στην παρούσα μελέτη, τα στελέχη *Aspergillus* παρουσίασαν την ίδια MIC στην αμφοτερικίνη Β (1 mg /L), που ταξινομούνται ως ευαίσθητα (Cantón et al, 2009), υποδεικνύοντας παρόμοια δράση της αμφοτερικίνης Β. Ωστόσο, το νέο in vitro μοντέλο αποκάλυψε διαφορές στην αποτελεσματικότητα της αμφοτερικίνης Β για τα τρία είδη *Aspergillus*, με την ακόλουθη σειρά: *A. fumigatus*>*A. flavus*>*A. terreus*. Αυτά τα ευρήματα είναι σε συμφωνία με προηγούμενες συγκριτικές μελέτες σε ζώα, όπου η θεραπεία με αμφοτερικίνη Β ήταν περισσότερο αποτελεσματική έναντι πειραματικών λοιμώξεων από *A. fumigatus* από ότι έναντι λοιμώξεων από *A. flavus* και λιγότερο αποτελεσματική έναντι πειραματικών λοιμώξεων από *A. terreus* (Odds et al, 1998; Walsh et al, 2003).

Στο νέο in vitro μοντέλο, η κασποφουγκίνη δεν έδειξε σημαντικές δοσο-εξαρτώμενες αλλαγές στην παραγωγή γαλακτομαννάνης από τα τρία είδη *Aspergillus* που μελετήθηκαν. Τα επίπεδα γαλακτομαννάνης μπορεί να παραμένουν σε υψηλά επίπεδα παρά την αντιμυκητιακή δράση της κασποφουγκίνη. Έτσι, για την φαρμακοδυναμική μελέτη των δράσεων της κασποφουγκίνης και των άλλων εχινοκανδίνων, μέτρηση της παραγωγής γαλακτομαννάνης δεν φαίνεται να είναι κατατοπιστική όπως βρέθηκε προηγουμένως σε in vitro και in vivo πειράματα (Petraitiene et al, 2002). Η PCR πραγματικού χρόνου έδειξε διαφορές στην δράση της κασποφουγκίνης μετξύ των ειδών.

ΣΥΜΠΕΡΑΣΜΑ

Συμπερασματικά, στην παρούσα διατριβή, αναπτύχθηκε ένα νέο in vitro PKPD μοντέλο το οποίο προσομοιώνει τη ανθρώπινη φαρμακοκινητική τριών αντιμυκητιασικών

φάρμακων που χρησιμοποιούνται στην θεραπεία της ασπεργίλλωσης (βορικοναζόλη, αμφοτερικίνη Β, κασποφουγκίνη) και αξιολογεί την αποτελεσματικότητά τους ενάντι το πιο κοινών ειδών *Aspergillus* (*A fumigatus*, *A. flavus*, *A. terreus*). Η in vitro PKPD ανάλυση της βορικοναζόλης έδειξε ότι απαιτείτε μεγαλύτερη έκθεση φαρμάκου για να αναστείλει τον *A. terreus* και *A. flavus* από ότι τον *A. fumigatus*. Επίσης, το νέο μοντέλο έδειξε φαρμακοδυναμικές διαφορές στη δράση της αμφοτερικίνης Β μεταξύ των τριών ειδών *Aspergillus* που δεν αντανακλώνται από τα MICs. Αυτές οι διαφορές μπορεί να οφείλονται σε διαφορές στις δόσο- και χρόνο-εξαρτώμενες ανασταλτικές/μυκητοκτόνες και μετα-αντιμυκητιακές δράσεις της αμφοτερικίνης Β εμφανίζοντας την μεγαλύτερη δραστικότητα έναντι *A. fumigatus* και την μικρότερη έναντι *A. terreus*. Σχετικά με κασποφουγκίνη, τα επίπεδα γαλακτομαννάνης παραμένουν σε υψηλά επίπεδα σε όλες της συγκεντρώσεις με αποτέλεσμα να μπορεί να περιγράψει την αντιμυκητιακή δράση της κασποφουγκίνης. Η χρήση του κονιδιακού ισοδύναμου με PCR πραγματικού χρόνου θα μπορούσε να βοηθήσει στην μελέτη της φαρμακοδυναμικής των εχινοκανδινών.

Τέλος, το νέο μοντέλο χρησιμοποιήθηκε για την προσομοίωση συνδυαστικής θεραπείας βορικοναζόλης+αμφοτερικίμης Β που συχνά χρησιμοποιείται στην θεραπεία της υποτροπιάζουσα ασπεργίλλωσης. Η μελέτη του συνδυασμού με το νέο μοντέλο έδειξε δόσο-εξαρτώμενες φαρμακοδυναμικές αλληλεπιδράσεις με τον συνδυασμό δοσολογιών που αντιστοιχούσαν στα ελεύθερα επίπεδα των δυο φαρμάκων μετά από χορήγηση των ενδεδειγμένων δόσεων να είναι συνεργικός. Επομένως, ο συνδυασμός βορικοναζόλη+αμφοτερικίνη Β μπορεί να αυξήσει την δράση της αντιμυκητιακής θεραπείας ενώ η παρακολούθηση των επιπέδων της συνδυαστικής θεραπείας θα μπορούσε να οδηγήσει στην αύξηση των συνεργικών αλληλεπιδράσεων έναντι των ασπεργίλλων .

2.5. References

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